EFFECT OF HERBAL EXTRACT ON RAT BONE MARROW STROMAL DERIVED OSTEOBLAST GROWTH AND DIFFERENTIATION *IN VITRO*

POON CHI TAT

FACULTY OF ENGINEERING
UNIVERSITY OF MALAYA
KUALA LUMPUR
2013

EFFECT OF HERBAL EXTRACT ON RAT BONE MARROW STROMAL DERIVED OSTEOBLAST GROWTH AND DIFFERENTIATION *IN VITRO*

POON CHI TAT

DISSERTATION SUBMITTED IN IN FULFILMENT OF THE REQUREMENTS FOR THE DEGREE OF MASTER OF ENGINEERING SCIENCE

FACULTY OF ENGINEERING
UNIVERSITY OF MALAYA
KUALA LUMPUR
2013

UNIVERSITI MALAYA
PERAKUAN KEASLIAN PENULISAN

Nama: POON CHI TAT

No. Pendaftaran/Matrik: KGA 080048

Nama Ijazah: Sains Kejuruteraan

Tajuk Disertasi ("Hasil Kerja ini"): Kesan Ekstrak Herba atas Pertumbuhan Osteoblas Perolehi daripada Stroma Sum-sum Tulang

Bidang Penyelidikan: Kejuruteraan Tisu

Saya dengan sesungguhnya dan sebenarnya mengaku bahawa:
(1) Saya adalah satu-satunya pengarang/penulis Hasil Kerja ini;
(2) Hasil Kerja ini adalah asli;
(3) Apa-apa penggunaan mana-mana hasil kerja yang mengandungi hakcipta telah dilakukan secara urusan yang wajar dan bagi maksud yang dibenarkan dan apa-apa petikan, ekstrak, rujukan atau pengeluaran semula daripada atau kepada mana-mana hasil kerja yang mengandungi hakcipta telah dinyatakan dengan sejelasnya dan secukupnya dan satu pengiktirafan tajuk hasil kerja tersebut dan pengarang/penulisnya telah dilakukan di dalam Hasil Kerja ini;
(4) Saya tidak mempunyai apa-apa pengetahuan sebenar atau patut semunasabahnya tahu bahawa penghasilan Hasil Kerja ini melanggar suatu hakcipta hasil kerja yang lain;
(5) Saya dengan ini menyerahkan kesemua dan tiap-tiap hak yang terkandung di dalam hakcipta Hasil Kerja ini kepada Universiti Malaya ("UM") yang seterusnya mula dari sekarang adalah tuan punya kepada hakcipta di dalam Hasil Kerja ini dan apa-apa pengeluaran semula atau penggunaan dalam apa jua bentuk atau dengan apa juga cara sekalipun adalah dilarang tanpa terlebih dahulu mendapat kebenaran bertulis dari UM;
(6) Saya sedar sepenuhnya sekiranya dalam masa penghasilan Hasil Kerja ini saya telah melanggar suatu hakcipta hasil kerja yang lain sama ada dengan niat atau sebaliknya, saya boleh dikenakan tindakan undang-undang atau apa-apa tindakan lain sebagaimana yang diputuskan oleh UM.

Tandatangan Calon Tarikh

Diperbuat dan sesungguhnya diakui di hadapan,

Tandatangan Saksi Tarikh

Nama:
Jawatan:

UNIVERSITI MALAYA
ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: POON CHI TAT

Registration/Matric No: KGA080048

Name of Degree: Master of Engineering Science

Title of Dissertation ("this Work"): Effect of Herbal Extract on Bone Marrow Derived Osteoblast Growth

Field of Study: Tissue Engineering

I do solemnly and sincerely declare that:
(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without thewritten consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date

Subscribed and solemnly declared before,

Witness's Signature Date

Name:
Designation:

ABSTRACT

Drynaria quercifolia (*D.quercifolia*) and *Justicia gendarussa* (*J.gendarussa*) are locally found herbs. Proper scientific studies were initiated to test the effects of the two plants and a pre-mixed herbal formulation (Bo-Gu-Cao formulation) with *J.gendarussa* as the main constituent. Rat bone marrow derived osteoblasts (rBMS-derived osteoblasts) were used as research subject in the present study. The effects of the herbal extracts on the cells were evaluated by comparing cell number and alkaline phosphatase (ALP) activity of the tested cells to control.

Samples of *D.quercifolia*, *J.gendarussa*, and pre-mixed Bo-Gu-Cao formulation were dried, powdered, and extracted with solvents of different polarities. Herbal extracts were reconstituted in media (at a concentration of 100 µg/ml) and subjected to proliferation rate, ALP activity evaluation, and staining of calcium deposition. Bone marrow stromal cells were isolated from tibias and femora of Sprague Dawley rats and subsequently induced to osteogenic differentiation. The cells were identified as bone marrow stromal cells prior to osteogenic differentiation by standard stem cell characterization. Cells were incubated with herbal extracts and evaluated with proliferation rate and ALP activity. Statistical analysis was performed by using One-Way ANOVA test to compare the means of samples and control values.

A total of 12 herbal extracts were obtained from the two herbs and the pre-mixed herbal formulation. Bone marrow stromal cells were identified as the microscopic observation confirmed attachment to plastic surface and fibroblastic morphology, characterized by CD90+, CD45- and CD31-, and the cells were able to be induced to osteoblasts, chondrocytes, and adipocytes. Calcium deposition by rBMS derived osteoblasts was stained by Alizarin Red S staining.

Water extract of *D.quercifolia* and water extract of *J.gendarussa* and Bo-Gu-Cao formulation consistently induced a significant enhancement on rBMS derived osteoblast proliferation on day 7 and day 14 of incubation. ALP activity was enhanced significantly in the cells incubated with water extract of *D.quercifolia* and *J.gendarussa* for 7 days and 21 days. The dose dependency effect evaluation had shown the optimum dosage for *D.quercifolia* water extract, while there is no dose dependency effect found on water extract of *J.gendarussa*. The results of the present study reveals that the medicinal herbs used in this study possess certain substance/s that can enhance osteoblast proliferation as well as differentiation and can be postulated in aiding bone healing.

It can be concluded that water extract of *D.quercifolia* and *J.gendarussa* are the potential herbal extracts to be further investigated. Isolation of active compounds from *D.quercifolia* and *J.gendarussa* are to be done and effects of the active compounds on osteoblasts are to be evaluated.

ABSTRAK

Drynaria quercifolia (*D.quercifolia*) dan *Justicia gendarussa* (*J.gendarussa*) merupakan herba tempatan. Kajian saintifik yang sesuai telah dijalankan untuk mengkaji kesan dua tumbuhan tersebut dan formulasi herba pracampuran (formulasi Bo-Gu-Cao) yang mengandungi *Justicia gendarussa* sebagai konstituen utama. Osteoblas yang berasal daripada sumsum tulang tikus (osteoblast terbitan rBMS) telah digunakan sebagai subjek penyelidikan dalam kajian ini. Kesan-kesan ekstrak-ekstrak herba pada sel telah dinilai secara membandingkan bilangan pertumbuhan sel dan aktiviti fosfatas alkali (ALP) sel-sel yang diuji dengan kawalan.

Sampel-sampel *D.quercifolia*, *J.gendarussa,* dan formulasi Bo-Gu-Cao pracampuran telah dikeringkan, dikisarkan, dan diekstrak menggunakan pelarut dengan kekutuban yang berlainan. Ekstrak herbal telah dikonstitusi semula dalam medium (pada konsenterasi 100 μg/ml) dan dikenakan kadar proliferasi, penilaian aktiviti ALP, dan pewarnaan endapan kalsium. Sel-sel stromal sumsum tulang telah diambil daripada tibia dan femora tikus Sprague Dawley dan seterusnya didorong ke pembezaan osteogenik. Sel-sel telah dikenalpastikan sebagai sel sumsum tulang stromal sebelum pembezaan melalui kaedah-kaedah pencirian sel stem yang standard. Sel-sel telah diinkubasi dengan ekstrak herba dan dinilai dengan kadar pertumbuhan dan aktiviti ALP. Analisis statistik telah dilakukan dengan menggunakan ujian *One-Way ANOVA* untuk membandingkan min bagi nilai sampel dengan kawalan.

Sejumlah 12 ekstrak herba telah diperoleh daripada dua herba berkenaan dan formulasi herba pracampuran. Sel-sel stromal sumsum tulang telah dikenalpastikan dengan pemerhatian mikroskopik pelekatan sah pada permukaan plastik dan morfologi fibroblastik, dicirikan oleh CD90+, CD45, dan CD31, dan sel-sel dapat membeza kepada osteoblas, kondrosit, dan adiposit. Pemendapan kalsium oleh osteoblas terbitan rBMS diwarnakan oleh pewarnaan Alizarin Red S.

Ekstrak air bagi *D.quercifolia* dan ekstrak air bagi *J.gendarussa* dan formulasi Bo-Gu-Cao secara konsisten mendorong peningkatan yang signifikan pada proliferasi osteoblast terbitan rBMS pada hari ke-7 dan hari ke-14 inkubasi. Aktiviti ALP telah dipertingkatkan secara signifikan pada sel-sel yang diinkubasi dengan ekstrak air *D.quercifolia* dan *J.gendarussa* untuk 7 hari dan 21 hari. Penilaian kesan kebergantungan dos menghasilkan dos optimum bagi ekstrak air *D.quercifolia*, manakala tiada kesan bergantung dos ditemui pada ekstrak air *J.gendarussa*. Hasil kajian ini mendedahkan bahawa herba perubatan yang digunakan dalam kajian ini mempunyai bahan tertentu yang boleh meningkatkan pertumbuhan osteoblas serta pembezaan dan seterusnya berkemungkinan mampu mempercepatkan penyembuhan tulang.

Dapatlah disimpulkan bahawa ekstrak air *D.quercifolia* dan *J.gendarussa* berpotensi untuk dikaji selanjutnya. Pemencilan sebatian-sebatian aktif daripada *D.quercifolia* dan *J.gendarussa* perlu dilakukan dan kesan-kesan sebatian-sebatian aktif itu pada osteoblas perlu dinilai.

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincere gratitude to my supervisor, Professor Ir. Dr. Wan Abu Bakar bin Wan Abas and my co-supervisor, Associate Professor Dr Kim Kah Hwi for their guidance, knowledge and support that allow me to finish this research project successfully. Very special thanks to them for their generous comment, patience and the fully trust they showed to me throughout the research project.

Many thanks go to Dr Belinda Murphy as the laboratory supervisor of Tissue Engineering Laboratory for giving me so much guidance and support throughout the study. She has given me so many ideas on how I was going to make my result presentable and can be published in ISI listed journals.

I am also very grateful to Mr.Johgalingam, a senior technician in Department of Physiology, Faculty of Medicine. He taught me how to sacrifice the rat and showed me the bone marrow isolation procedure before I dared to handle rat for this study. Without him, this study can never be started as the rat bone marrow was the most important research model in this study.

Not to be forgotten, thanks to Mr. Lim Kok Hong for supplying the herb and giving me information about the herb. Thanks to Dr Sugumaran, a taxonomist from Rimba Ilmu, for identifying the medicinal herbs.

And also to my fellow labmates, seniors and friends, thanks for all your wonderful support and advice given to me throughout my research project.

A special thanks to my fiancée, Chan Suet Oie, for giving me so much support, cheers me up when i was down because of obstacles in this project and shares my happiness when finding a breakthrough in the study.

Last but not least, I would like to express my gratitude to my beloved family for their support and encouragement. Without them, my study could never been completed.

And also thanks to all the people who had participated in making this research project successful.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
β-GP	Beta gylcerophosphate
BMSC	Bone marrow stromal cell
BMD	Bone mass density
CCK-8	Cell counting kit 8
CT	Computed topography
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle's Medium
DMEM/F12	Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham
FCS	Fetal calf serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
HSC	Hematopoietic stem cell
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cell
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)
OD	Optical density
OM	Osteogenic medium
PBS	Phosphast buffered saline
pNPP	p-nitrophenol phosphate
RBC	Red blood cell
RBMS	Rat bone marrow stromal
RGD	Arginine-glycine-aspartate
SD	Standard deviation
SD rat	Sprague Dawley rat

TGF-β	Transforming growth factor beta
WBC	White blood cell
WST-8	[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium

CHAPTER 1:

INTRODUCTION

1.1 BONE FRACTURE

1.1.1 Bone fracture: Overview

Fracture is defined by a break, rupture or crack in bone or cartilage. Despite the efficiency of its self-repairing ability, bone fracture is a very common and general health problem. Recovery of fracture takes time, and usually accompanied with pain. Its occurrence could be found on a person regardless of age and gender, due to various reasons, for example a sudden appearance of a load that exceeds bone strength (e.g. impact received by a person trauma) or a gradually accumulation of damage at a rate that cannot be repaired by the bone tissue itself. For those who have low bone density, such as peoples with osteoporosis, bone tumor, or osteogenesis imperfecta are more susceptible to bone fracture due to lacking of bone strength (Liao *et al.*, 2005; Lane, 2006). Fracture occurs mainly due to weaken bone as a result of the above medical conditions is termed pathologic fracture. Fractures caused by osteoporosis affect 50 % women and 20 % men over the age of 50 (Cummings and Melton, 2002).

There are many types of fractures, but mainly categorized to displaced, non-displaced, open, and closed (Alms, 1961). The way the bone breaks can be either displaced or non-displaced fractures. In a displaced fracture, the bone has broken into two or more parts and moves so that the two ends are separated. Comminuted fracture is when the bone is in more than two pieces. When a fracture is non-displaced, that means the bone is broken either part or all of the way through but still remains in place, and sometimes it will cause the bone its natural shape. A closed fracture is when the bone breaks but the skin is still intact. The broken bone does not penetrate skin. Thus there are neither punctures nor pen wound seen in this case. An open fracture requires immediate medical handling as the bone breaks through the skin. This is an important

distinction from a closed fracture because with an open fracture there is a risk of a deep bone infection. An operation is often required to clean the area of fracture to avoid infection.

Bone fracture causes problems and difficulties in daily life, depending on the extent of the bone fracture. Normally bone fracture comes with pain and inability to move freely at the fracture part. This would widely affect the working performance and daily activities such as doing household chores, exercise and even simple walking in the worst case. Thus a fast healing and more convenient way to treat bone fracture are needed.

1.1.2 BONE ANATOMY

Bones are organs which build up the skeleton in vertebral animal. They work together with other skeletal tissues like tendons, cartilage, ligaments and muscles in vertebral animal movement. Bone also provides protection to various internal soft internal organs of the body as well as maintains posture. Other functions of bone in human are production of red and white blood cells and become reservoir for minerals such as calsium and phosphate in maintaining homeostasis in body (Taichman, 2005). Composition of bone include dense specialized connective tissue that consists of osteogenic cells such as osteocytes, osteoblasts, osteoclasts and osteoprogenitor cells and mineralized extracellular matrix. When the osteoblasts have surrounded themselves with their secreted matrix, they will later on mature into osteocytes. Osteoclasts are multinucleated phagocytic cells derived from bone marrow that responsible for bone resorption (Hughes and Porter, 1997).

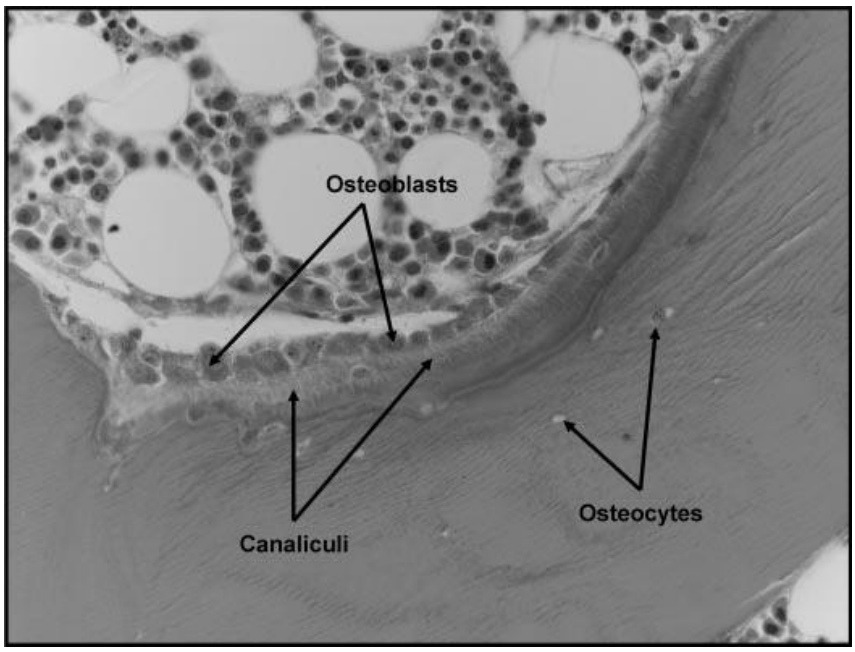


Figure 1.1: Osteoblasts synthesize proteinaceous matrix and become osteocyte after surrounded with the matrix (adopted from Clarke, 2008).

The bone surface is covered by periosteum that consists of an outer fibrous layer that resembles other dense connective tissue, and an inner cellular layer containing osteoprogenitor cells or periosteal cells. The periosteal cells are capable of differentiating into osteoblasts under appropriate stimulus (Ross *et al.*, 1995). The endosteum is the lining tissue of both the compact bone facing the marrow cavity and the trabeculae of spongy bone within the cavity. This endosteum consists of fibroblastic flattened cells or called endosteal cells, that are capable of differentiating into osteoblast under appropriate stimulus too (Ross *et al.*, 1995). Mineralized extracellular matrix is essential for the structural stiffness and strength of bone. It is formed during the mineralization of bone tissue. The extracellular matrix comprises 20 % to 40 % organic (type I collagen contributes 90 % of it, the rest comsists of osteocalcin, osteonectin proteoglycans and glycosaminoglycans and lipids) and 50 – 70 % inorganic substances which primarily consists of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_3$], with small amount of carbonate, magnesium, and acid phosphate. The bone hydroxyapatite crystals are more water-soluble than geologic hydroxapatite crystals, thus they play important role in

mineral metabolism. The mineralized extracellular matrix becomes extremely hard when fortified by the presence of calcium phosphate minerals in the form of hydroxyapatite crystals.

Bone can be classified due to different categories. In term of bone tissue type, bone can be classified as either Compact (Dense) or Spongy (Cancellous). Compact bone is the dense bone tissue that forms the outside of the bone and also outline of the bone, whereas spongy bone is a bone trabecular meshwork which has the appearance of a sponge meshwork in the interior of the bone. Bone marrow and blood vessels occupy the meshwork spaces. Bone can be classified based on the shapes. There are long bones, short bones, flat bones, and irregular bones. Long bones are those that are longer than they are wide, and grow primarily by elongation of the diaphysis, with an epiphysis at the ends of the growing bone. The ends of epiphyses are covered with a hyaline cartilage, which function is to stop bone ends from rubbing each other and acts as an 'absorber' to shock. The longitudinal growth of long bones is a result of endochondral ossification at the epiphyseal plate. Bone marrow in young developing individuals are called red bone marrow as it consists of developing blood cells in different stages of development and a network of reticular cells and fibers that serve as a supporting framework for the developing blood cells and blood vessels. In adult individuals, where when the rate of blood cell formation has diminished, adipose cells are the majority in the marrow cavity, which is called yellow marrow. Under certain circumstances, such as excessive blood loss, the yellow marrow can revert to red marrow. Bone growth in length is stimulated by the production of growth hormone (GH), a secretion of the anterior lobe of the pituitary gland. The long bones include the femurs, tibias, and fibulas of the legs, the humeri, radii, and ulnas of the arms, metacarpals and metatarsals of the hands and feet, and the phalanges of the fingers and toes. The long bones of the human leg comprise nearly half of adult height.

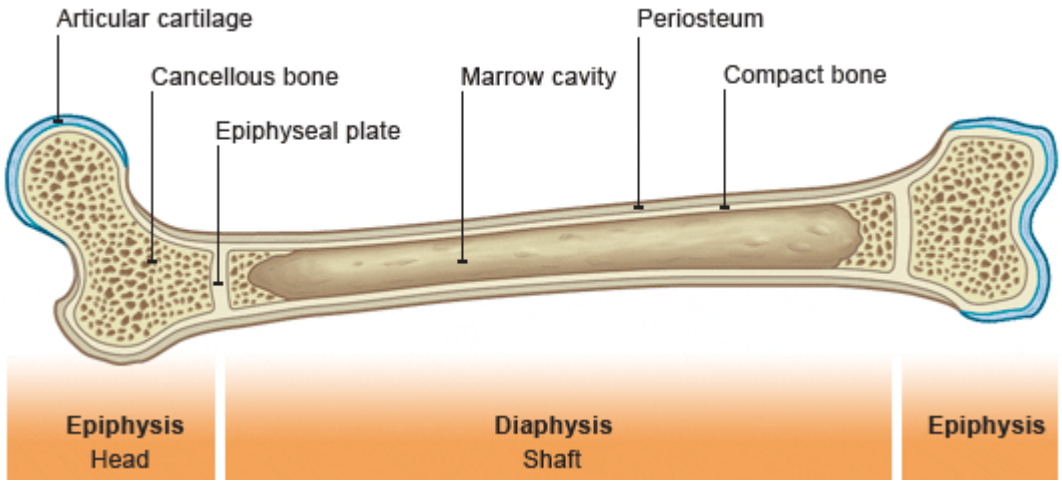


Figure 1.2: Long bone anatomy.

Short bones are approximately as wide as their length. The main function of short bones is providing support and stability as they spread load efficiently. Tarsal and carpals are examples of short bones. Ribs and cranium are categorized as flat bones. Flat bones are strong, flat in shape to cover vital organs such as brain and lung. Besides, they provide attachment base of muscle, such as on scapula. Flat bones are important reservoir of red blood cells as highest number of red blood cells is formed in flat bones. Other none uniform shape bones are named as irregular bones. Vertebrae and face fall into this category. The basics of bone microstructure include both Volkmann's & Haversian canals- basic nutritional and blood supplies for the bone. At the micro level, the basic structural unit of compact bone is osteon. The cortical osteons are called Haversian systems. Haversian systems are cylindrical in shape, are approximately 400 mm long and 200 mm wide at their base, and form a branching network within the cortical bone (Eriksen *et al.*, 1994). Haversion canal contains small blood vessels responsible for blood supply to osteocytes and nerves. Osteon tends to run parallel to the long axis of a bone. The canaliculi are arranged in a radial fashion with respect to the canal and serve for the passage of substances between the adjacent osteocytes and also the blood vessels. Between the osteons is either remnant of previous osteonal

lamellae called interstitial lamellae or the lamellar bone. Circumferential lamellae follow the entire inner and outer circumferences of bone. Volkmann's canals are channels in lamellar bone through which blood vessels and nerves travel from the periosteal and endosteal surfaces to reach the osteonal canal and from one osteonal canal to another.

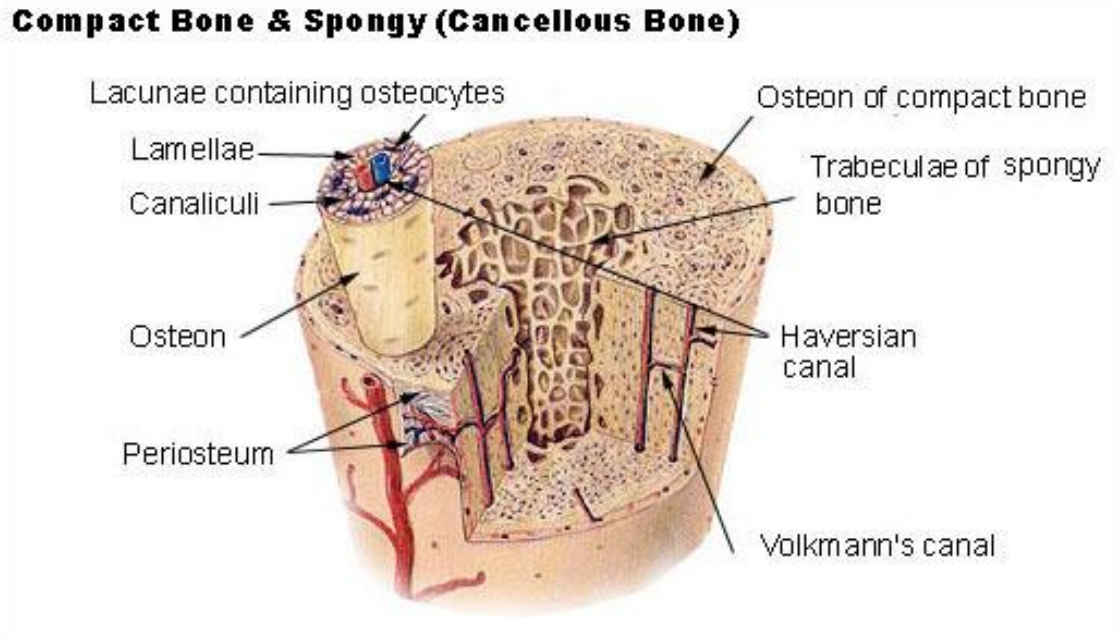


Figure 1.3: Microscopic structure of bone.

1.1.3 Bone physiology: Formation and repair

There are two different ways of development of bone or ossification: endochondral ossification and intramembranous ossification. A clear difference between the two types of bone development is that endochondral ossification involves a cartilage model to serve as the precursor of the bone, whereas intramembranous ossification does not involve a cartilage precursor and the bone is formed by a simpler method.

Intramembranous ossification occurs as early as around the eighth week of gestation in human. At this early stage of human embryonic development, a cartilage

model of skeleton is formed from the mesenchyme. In the further fetal development of long bones, a rim of primitive bone is first laid down in layers over the middle of the shaft by osteoblasts arising from the overlying periosteum, and subperiosteal bone formed in this way soon extends up and down the shaft (diaphysis). Pale-staining elongate mesenchymal cells are observed, and migrate and aggregate in the specific areas where bone is destined to form. This newly formed tissue is subsequently becomes more vascularized and the mesenchymal cells aggregate and become larger and rounded. At this stage, the mesenchymal cells are said to be osteoblastic-like and containing cytoplasm that are more basophilic with an apparent Golgi apparatus. These osteoblastic-like cells or the differentiated osteoblasts will then migrate to the membranes and secrete more and more bone matrix collagen and proteoglycans. The deposition of bony matrix separates the cells from one another. However, they remain interconnected with the cytoplasmic processes. Bone calcification occurs after bone matrix appears denser than the surrounding tissue due to the abundant collagen content. When the osteoblasts are surrounded by matrix they are called osteocytes. Generally, osteocytes are formed when they are trapped in the lacunae and canaliculi. Intramembranous ossification involves the replacement of sheet-like connective tissue membranes with bony tissue. Bones formed in this manner are called intramembranous bones. They include certain flat bones of the skull and some of the irregular bones. The future bones are first formed as connective tissue membranes. Osteoblasts migrate to the membranes and deposit bony matrix around them.

Proliferation of surrounding primitive cells gives rise to a population of osteoprogenitor cells which come into apposition with the initially formed spicules. They differentiate into osteoblasts and add more bone matrix to the developing spicules until a continuous bone matrix is formed. In other words, these osteoprogenitor cells provide a constant source of osteoblasts for growth of the bone spicules, which in turn

lay down bone matrix in successive layers, giving rise to woven bone. This immature woven bone is characterized by interconnecting spaces occupied by connective tissue and blood vessels (Figure 1.5) (Ross *et al.*, 1995).

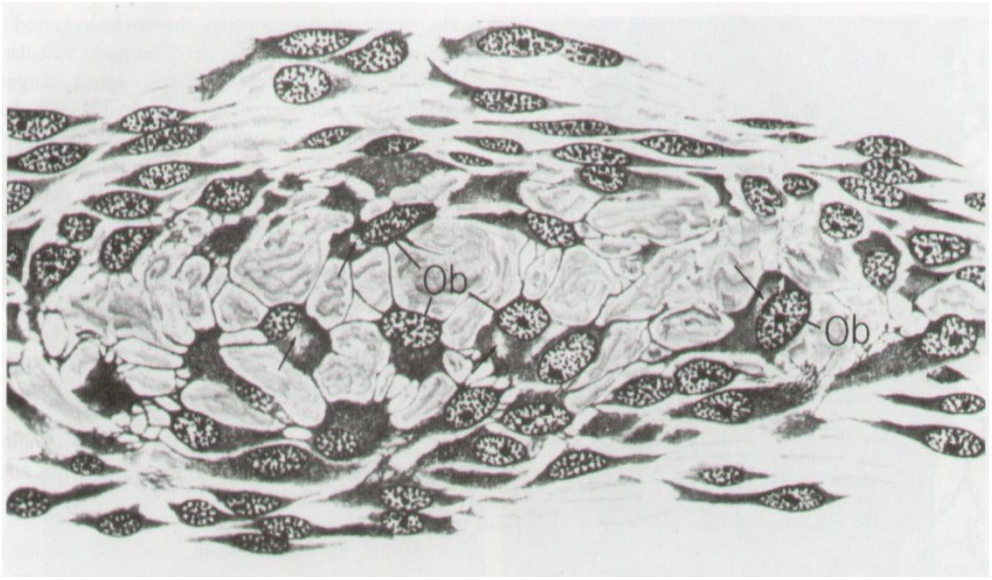


Figure 1.4: Intramembranous ossification (Ross *et al.*, 1995).

Endochondral ossification is a complex, multistep process requiring the sequential formation and degradation of cartilaginous templates for the developing bones. Most of the bones of the skeleton are formed in this manner and these bones are called endocondral bones. Principally the process replaces hyaline cartilage with bony tissue. The process begins with the proliferation and aggregation of mesenchymal cells at the site of bone formation, which later on differentiate into chondroblasts that in turn produce cartilage matrix and subsequently a hyaline cartilage model is established and it is to be the future bone. The perichondrial cells in the midregion of the cartilage model start to be infiltrated with osteoblasts and blood vessels until a thin layer of osteogenic tissue or periosteal bone is formed around the cartilage model. The cartilage in the epiphyses continues to grow so the developing bone increases in length. Later, usually after birth, secondary ossification centers form in the epiphyses. At this stage,

chondrocytes in this midregion of the cartilage model become hypertrophic and begin to synthesize alkaline phosphatase (ALP) that lead to surrounding cartilage matrix calcification. Later on, chondrocytes in the cartilage model die off as a result of inhibited diffusion by calcified cartilage matrix. Ossification in the epiphyses is similar to that in the diaphysis except that the spongy bone is retained instead of being broken down to form a medullary cavity. Concomitantly, blood vessels grow through the thin bone collar layer to vascularize the cavity. This vascularization allows further migration of periosteal cells into the cavity and bone marrow is then formed. From the break-down of the calcified cartilage, some remain as irregular spicules, into which later on osteoblasts lay down bone matrix on the spicule framework and with time developed into bone. Histologically, calcified cartilage tends to be basophilic (purplish blue), whereas bone is distinctly eosinophilic (pinkish) (Ross *et al.*, 1995).

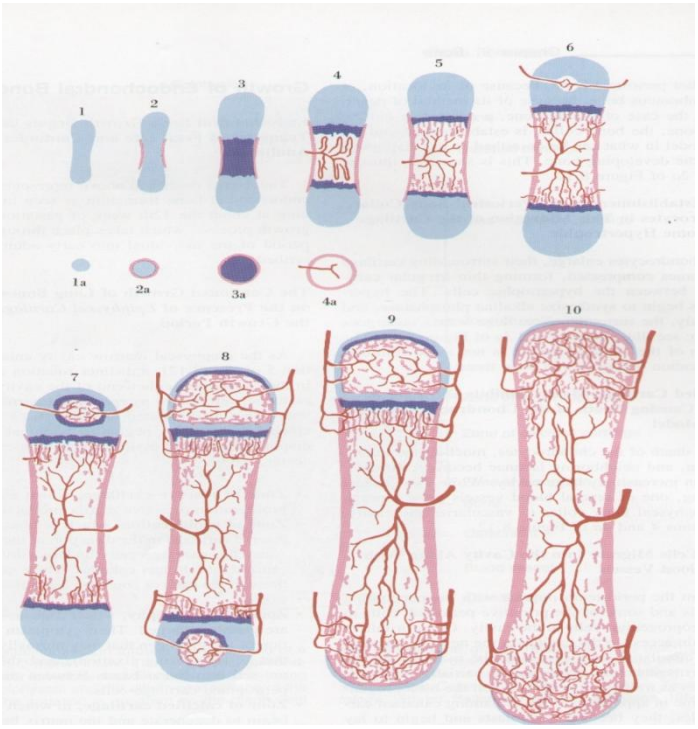


Figure 1.5: Endocondral ossification (Ross *et al.*, 1995).

Bone healing requires adequate blood supply. Fracture stimulates the release of growth factors that promote angiogenesis and vasodilatation. Initial response of bone fracture is similar to any occurrence of injury. Tissue destruction and hemorrhage are found at the fracture area. This is followed by acute inflammation reaction which characterized by neutrophil invasion and macrophage debridement. Fibroblasts and capillaries proliferation and growth occurs subsequently, and followed by tissue granulation and finally cartilage or callus formation at the site of injury. Callus formation helps to align and bind together the fractured bone. At the same, osteoprogenitor cells of periosteum proliferate and differentiate into osteoblasts and begin to deposit new bone matrix on the outer surface of the bone at some distance from the fracture. Then, these cells progress toward the fracture site until new bone forms a bony sheath over the fibrocartiliginous callus. This newly-formed bone will then invade the callus and lay down new bone matrix within the callus, which gradually replacing the callus, as which happen in endochondral ossification. Similarly, in the marrow cavity, endosteal proliferation and differentiation giving rise to medullary bone grow from the both ends of the fracture toward the center, unite and spongy bone is then formed. This spongy bone is gradually replaced by compact bone, and the bony callus is removed and remodeled by the action of osteoclasts. The process could take from weeks to several months, depending on the heath condition of the individual and severity of the fracture (Ross *et al.*, 1995).

Bone physiology is a dynamic phenomenon. Bone remodeling occurs throughout an individual life whereas mature bone cell is replaced by new formed bone cell as a result of the physiological activity of the bone cells at the endosteal surface of bone. Unlike modeling, bone remodeling cannot cause large changes in bone structure at a given site (Kobayashi *et al.*, 2003). In bone remodeling, osteoblasts and osteoclasts do not act independently but are coupled. Bone resorption and formation are two major

processes in bone remodeling. They do not occur along the bone surface at random but the processes are co-ordinated in reshaping or replacement of bone following fractures or micro-damage to bone which occurs in normal daily activity. In healthy individuals, net effect of bone resorption and formation occur at the same rate as to maintain the current amount of bone mass and structure. Imbalance of the processes could happen due to aging or pathological condition such as osteoporosis, result in net bone loss.

There are five stages in bone remodeling. The resting state of the bone surface is referred as quiescence. Activation requires the recruitment of osteclasts to a bone surface and signal coupling of osteoblasts (Roodman, 1999). After activation, resorption takes place which removes bone by osteoclasts. Reversal is the process by which osteoclasts stop removing bone and osteoblasts fill the defect. Finally, formation is the laying down of bone by osteoblasts.

1.1.4 Risk factors of bone fracture

There are factors or a combination of factors increase the risk of bone fracture e.g. age, low bone mass density (BMD), body weight, gender, pathological condition, nutrition problem and history of prior fractures (Lefauveau and Fardellone, 2004; Olszynski *et al.*, 2004; White *et al.*, 2006; Benetos *et al.*, 2007).

Fractures prevalence in community is in bimodal pattern, with peak at young and elderly. Bone density and muscle mass both decrease with age. Vision and balance problems are common in older age and these will increase the risk of falling which could result in fracture. Inactive lifestyle when aging causes muscles tending to weaken. Fractures in children are common as well. The reported incidence of fractures in the United Kingdom in children ranges from 1.6 % per year to 3.6 % per year (Lyons *et al.*, 2000). Fractures in children are always associated with substantial trauma.

Women lose bone density at a faster rate than men do. This is largely due to drop significantly in estrogen levels and then increasing the risk of fractures (Riis *et al.*, 1996). However, men also can develop dangerously low levels of bone density.

Osteoporotic fracture may be the most concerned pathological fracture. About 44 million American men and women are affected by osteoporosis, accounting for 55 % of the population age 50 and older (Foundation, 2002). Osteoporosis is the most significant factor for fracture in elderly over 70 (Holroyd *et al.*, 2008). Other medical conditions may lead to fragile bones as well. These include endocrine disorders, such as an overactive thyroid, and intestinal disorders, which may reduce absorption of vitamin D and calcium (Basha *et al.*, 2000; Dhanwal, 2011). Recent studies have shown that type 1 and type 2 diabetes could be another risk factor for fracture (Merlotti *et al.*, 2010).

Long term consumption of medications, such as prednisone (Romas, 2008) and anti-epileptic drug (Dent *et al.*, 1970) can weaken bone. Corticosteroids can reduce bone density and increase the risk of fractures (Peel *et al.*, 1995). Bone density loss occurs rapidly after use of corticosteroids but is reversible (Romas, 2008). In some cases, certain drugs or the combination of medications can make you dizzy and more prone to falling.

Nutritional problems such as lack of calcium and vitamin D in diet lowers bone mass and increases risk of fracture (Rizzoli, 2008). Serious eating disorders, such as anorexia nervosa and bulimia, can damage skeleton by depriving body of essential nutrients needed for bone building.

Some habits such as **s**moking and drinking alcohol can interfere with the normal processes of bone building and remodeling, resulting in bone loss (Compston, 2007; Berg *et al.*, 2008).

1.1.5 Diagnosis and management of bone fracture

Patient history and physical examination are important in a diagnosis of bone fracture. An experienced and well-trained physician is needed to perform the above two clinical measures. Usually, physician utilizes a readily learned and consistently practiced manual examination for bone stability prior to X-ray. Imaging by X-ray is often performed to view the bone suspected of being fractured. Clinicians can usually recognize most fractures by examining the injury and taking X-rays. However, X-ray does not always helpful because some wrist fractures, hip fractures, and stress fractures are hardly recognized on an X-ray image. A computed tomograph (CT scan) may be performed in case the fracture is unfortunately unable to be diagnosed by X-ray alone. In these situations, physician may perform other tests, such as a computed tomography (CT) scan or magnetic resonance imaging (MRI). CT scan uses a combination of x-rays and computer technology to produce cross-sectional images of the body. A CT scan shows detailed images of any part of the body, including the bones, muscles, fat, and organs. Compared to X-ray image, CT scans are more detailed. MRI is a diagnostic procedure that uses a combination of large magnets, radiofrequencies, and a computer to produce detailed images of organs and structures within the body. This test is done to rule out any associated abnormalities of the spinal cord and nerves. CT scan, MRI, or angiogram may be needed to determine whether other tissues around the bone have been damaged. Generally, MRI is more effective than CT scan in term of identifying occult fractures, indicated in the study of (Lubovsky *et al.*, 2005)

As fracture happens, immobilization of the fracture of arms, legs, hands and feet is the common measure. Splinting the injury site with plaster or fiberglass is to immobilize the break. In most of the cases, splint is removed and replaced by a circumferential cast after few days in which edema at injury site has ceased. Edema at

injury site could cause a buildup of pressure under the cast, cause pain and the potential for damage to the tissues. Extra care has to be taken for injuries of the neck and back to protect the spinal cord from potential injury. Placing the injured person on a long board and in a neck collar is the normal practice by paramedics. An operation is needed when the surgeon thinks that the bone heals improperly. There might be the risk of the break moves out of place after it is aligned. Pins, plates, or rods are inserted into the bone to hold it in place until healing occurs. Some of these pieces of metal are permanent, and some are temporary until the healing of the bone is complete and another surgery is needed to remove at a later time.

In traditional bone-defect management, the use of autograft, allograft, vascularized fibula and iliac crest grafts, and other bone transport techniques are standard treatments. However, there are a lot of limitations on currently performed treatments (Finkemeier, 2002). First of all, the operation procedures for harvesting bone graft is expensive and contribute significant donor site morbidity associated with infection (Banwart *et al.*, 1995), pain (Heary *et al.*, 2002; Kim *et al.*, 2009; Schwartz *et al.*, 2009) and hematoma (Silber *et al.*, 2003). In addition, the bone graft is avascular which can lead to poor nutrient diffusion and can be problematic due to unpredictable bone resorption before osteogenesis is complete (Enneking *et al.*, 1980; Brown and Cruess, 1982).

Allografting introduces the risk of post-operative infections (such as HIV infection (Marthy and Richter, 1998)), and hepatitis C (Krajden *et al.*, 1995), chronic graft-versus-host response (Arora *et al.*, 2003) and may cause a lessening or complete loss of the bone inductive factors (Bostrom *et al.*, 2001). Vascularized grafts require sophisticated infrastructure and the operation procedure is often laborious and lengthy. These have lead to the need to develop bone regeneration alternatives that can be used

in the reconstruction of large orthopedic defects or bone implants that are much more mechanically stable and biocompatible.

1.2 TISSUE ENGINEERING

1.2.1 Concept of Tissue Engineering

In conventional conception, removal of a failed organ or tissue due to infection or injury is the normal practice especially when progressive condition of the damaged tissues and organs is found life threatening. However, this health management measure is not always suitable for all conditions. When there is damage or dysfunction of vital organs such as heart, brain, liver and kidney, removal of such organs is not appropriate as the organs are important to sustain life and the tissues are not able to regenerate. Besides that, removal of damaged limbs and other non-critical organs causes permanent physical deformity and disability that may lead to further life-threatening complications.

Artificial or prosthetic materials replacement therapies have been evolved in order to overcome the limitations of amputation. Such therapies provide at least partial restoration of the lost function such as installation of artificial or prosthetic materials as the replacement to amputated limbs. Organ transplantation is another choice for function restoration of a damaged organ. In organ grafting, although molecular and cellular events of immune response have been elucidated successfully to suppress the response against transplanted organ and to prolong graft survival and function, abnormal interactions of transplanted organ or graft with tissue at the new location have produced biological changes that may lead to cancer formation or other unexpected clinical complications (Mitruka *et al.*, 1997; Gulley *et al.*, 2003; Lindelof *et al.*, 2005). Difficulty in searching for a suitable donor is another major problem contributing to the high mortality rate for patients waiting for organ transplantation. When organ transplantation is found with limitations as above problems, biocompatible biomaterials are invented and able to be implanted in human body. The artificial heart valve (Dasi *et al.*, 2009) and total joint replacement (Diomidis *et al.*, 2012) therapies are two worth

mentioning examples. However, despite of the advances, techniques using implantable medical devices and biomaterials for replacing damaged organs or structures have produced problems such as displacement, fracture, erosion, and migration over time, as well as infection at implant-tissue interface. A second surgery is often required to remove the implanted material once the function of damaged organ has been restored. The second surgery increases the risk of site infection and gives rise to other clinical complications.

A new emerged field, called tissue engineering, applies the principles of biology and engineering to the development of reparative medicine – replacing damaged organ and restoration of the lost function. Nowadays tissue engineering researchers work toward an ultimate goal that living tissues and organs can be routinely assembled and reliably integrated into the body to restore, replace, or enhance tissue and organ functions. Application of tissue engineering to reparative medicine shows great promising future for the treatment of a large number of pathological conditions including birth defects, musculoskeletal disorders, Alzheimer's and Parkinson's diseases, diabetes, heart disease, liver and kidney failure, and spinal cord injuries which medical conditions of most of them are currently irreversible (Sipe, 2002). Regeneration of tissues which usually do not regenerate after maturation remains the main aim. Description of tissue engineering by Langer and Vacanti, 1993 indicates that the technology merges the fields of engineering, cell and molecular biology, materials science, and surgery, toward the development of biological substitutes that restore, maintain, and improve the function of damaged tissues and organs. Boyce and Warden, 2002 stated that tissue engineering provides novel combinations of cells, acellular biomaterials, drugs and gene products that may be designed, specified, fabricated, and delivered either simultaneously or sequentially as therapeutic agents.

The base concept of tissue engineering is manipulating the three major components of biological tissues - cells, the extracellular matrix and the signaling systems to improve or repair biological functions. It is important to know the interaction of each component in understanding of tissue engineering. Cells are the ground substances responsible for extracellular matrix secretion, with the presence of proper signaling systems that trigger differential activation of genes or cascades of genes whose secreted or transcriptional products are responsible for tissue formation and differentiation. Therefore, the model of tissue engineering concept consists of three main components: the cells, the scaffolds, and the signaling system (Figure 1.1). The scaffolds serve as mechanical support for cells growth; the cells are progenitor cells that can be differentiated into specific cell types, and the signaling system that can modulate cellular activities. When tissue engineering comes to medical application, the ideal scenes will be isolation of specific cells from a patient, following by growing them on a three-dimensional biomimetic and biodegradable scaffold under precisely controlled culture conditions and delivering the tissue construct to the defect site in the patient's body, thus allowing the tissue construct to organize and develop into a specific functional organ, while the scaffold degrades over time.

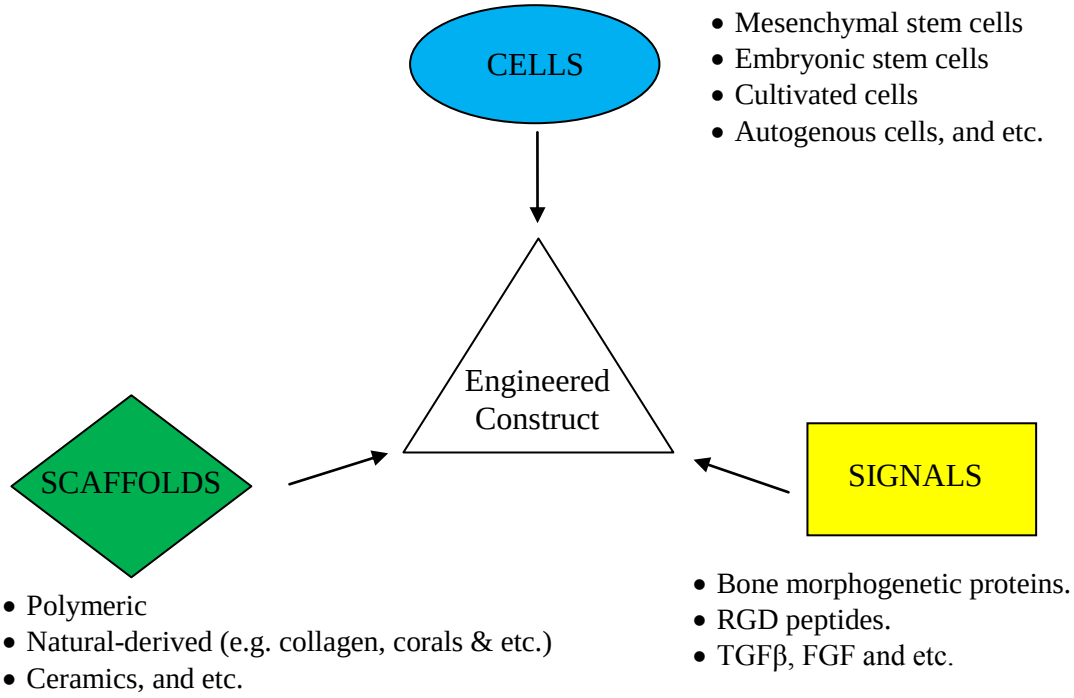


Figure 1.6: Idea of Tissue Engineering.

Pluripotent stem cells are those living cells that are capable of undergoing subsequent differentiation after being cultivated in-vitro. The cells are commonly accommodated to the scaffold and proliferated in situ. Stem cells can be induced to differentiate into desired cell type with the presence of proper signals, such as growth factors, chemicals or proteins. Stem cells can be obtained from different parts of the body and have been identified to be able to differentiate into specific type of cells that eventually may lead to tissue formation. There are excellent examples like embryonic stem cells recovered human blastocyst (Thomson *et al.*, 1998), satellite cells found in striated muscle (Renault *et al.*, 2002), keratinocytes of the skin (Olszewski *et al.*, 2005), and neuronal stem cells discovered in central nervous system (Alexanian and Kurpad, 2005), and cerebellum (Alcock *et al.*, 2007). Meanwhile, mesenchymal stem cells isolated from bone marrow are identified to be successfully differentiated into skin, cartilage, bone, adipose tissue, tendon, skeletal muscle and cardiomyocyte (Chaplan, 1991; Pittenger *et al.*, 1999; Alhadlaq and Mao, 2003; Alhadlaq and Mao, 2004; Croft

and Przyborski, 2004; Pittenger and Martin, 2004). Figure 1.2 depicts the classification of pluripotent stem cells in adult tissues (Fukuda, 2002).

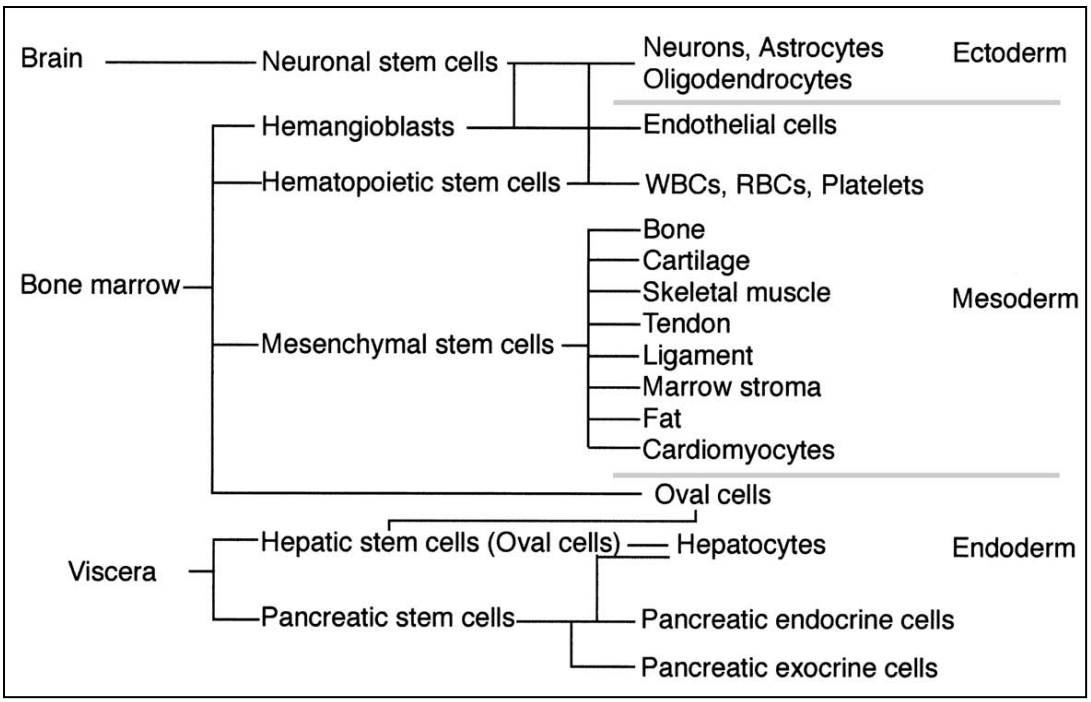


Figure 1.7: The classification of pluripotent stem cells in adult tissues.

1.2.2 Bone Mesenchymal Stem Cell (BMSC)

There are two types of stem cells found in bone marrow, namely the Hematopoietic Stem Cells (HSCs) and Mesenchymal Stem Cells (MSCs). HSCs are responsible for contributing progenitor cells for several lineages of hematopoietic cells, such as neutrophils, eosinophils, monocytes, T-lymphocytes, B-lymphocytes, erythrocytes and platelets whereas MSCs are cells for several types of connective tissues muscle, bone, cartilage, tendon, ligament and adipose tissues (Chaplan, 1991; Pittenger *et al.*, 1999), on the other hand. MSC are also known as Marrow Stromal Cells (Alhadlaq and Mao, 2004). MSC are an important model in tissue engineering research because of their characteristics - self-renewable and multipotent. In addition to the

differentiation into their natural derivatives, MSC have the potential to differentiate into other types of tissue-forming cells. These include hepatic (Peterson *et al.*, 1999; Ong *et al.*, 2006), renal (Poulsom *et al.*, 2003), pancreatic (Vija *et al.*, 2009), pulmonary (Spees *et al.*, 2007), cardiac (Clavel and Verfaillie, 2008), and neural cells (Jin *et al.*, 2003). (Figure 1.8)

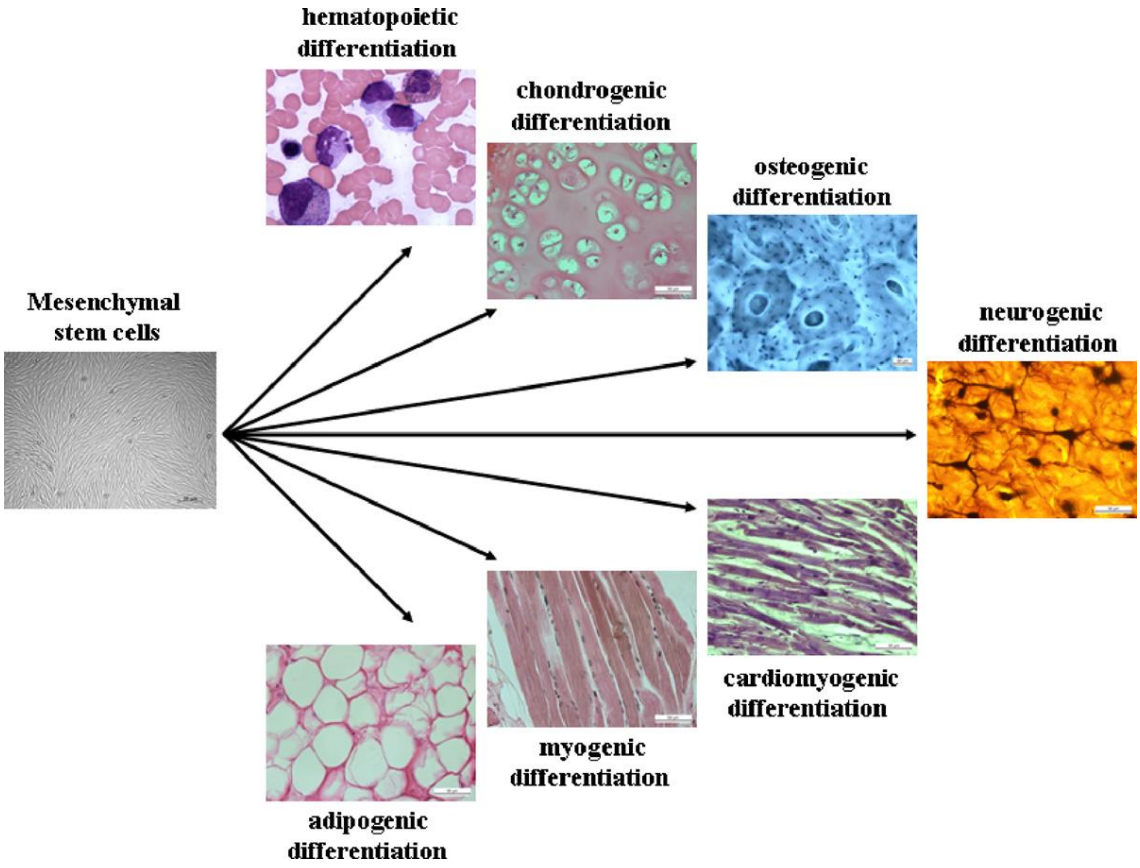


Figure 1.8: Multilineage potential of Mesenchymal Stem Cell (MSC). (Adopted from Danisovic *et al.*, 2012).

1.2.3 Osteogenic potential of BMSC

Bone marrow-derived fibroblastic colonies were first successfully isolated from guinea pig as described by Friedenstein *et al.*, 1970, based on the adherence of marrow-derived fibroblastic cells to the plastic substrate of the cell culture plate and a concomitant lack of adherence of marrow-derived hematopoietic cells. These fibroblastic cells were found to be able to become osteocytes when implanted into recipient animals (Friedenstein *et al.*, 1966). Similar cells were found in rabbit (Roostaeian *et al.*, 2006), rat (Grigoradis *et al.*, 1988; Poliard *et al.*, 1995), mouse (Qu *et al.*, 1998) and human (Haynesworth *et al.*, 1991) and were successfully isolated and induced to form osteogenic cells. And the fact is, bone marrow only consists of 0.001 % to 0.01 % of mesenchymal stem cells, but the high proliferation rate of these isolated mesenchymal stem cells enables them to be expanded over one billion-fold in culture (Haynesworth *et al.*, 1992). However, the marrow-derived MSC obtained by this technique are heterogeneous, which likely contains a variety of cells including fibroblasts, osteoblasts or osteoprogenitor cells, adipose cells, reticular cells, macrophages, endothelial and a fraction of hematopoietic stem cells (Seshi *et al.*, 2000). Consequently, this has led to the investigation to prepare primary cultures of bone marrow-derived MSC with more homogenous cell population using other more sophisticated isolation protocols as well as cytokines detection methods. For example, monoclonal antibodies detection (Haynesworth *et al.*, 1992; Jaiswal *et al.*, 1997), and cell specific markers expression (Seshi *et al.*, 2000).

Typically, demonstration of osteogenic differentiation potential of MSC in monolayer cultures obtained as described by Friedenstein *et al.*, 1970 can be achieved by incubating first-passage rat-derived MSC cultures in osteogenic medium for up to 4

weeks. To date, an osteogenic medium has been well-defined and widely used for this purpose.

Dexamethasone is a synthetic glucocorticoid which is found to be able to induce BMSC to differentiate into osteogenic cells (Qu *et al.*, 1998; Ogston *et al.*, 2002; Oshina *et al.*, 2007). However, this concentration-dependent stimulation (range from 10^{-7} M to 10^{-6} M) could result in bone loss (Iu *et al.*, 2005). According to Chang *et al.*, 2009 and Walsh *et al.*, 2001, an inhibitory effect of dexamethasone on more osteogenic precursor cells resulting in depletion of the osteogenic population and eventually bone loss occurred.

β-glycerolphosphate (β-GP) facilitates the matrix mineralization process by supplying organic phosphate to the culture medium. The matrix mineralization process involves a cell-mediated cleavage of this organic phosphate supplemented in the culture medium into inorganic phosphate by the cell membrane associated enzyme, ALP. However, the release of enzyme ALP by cells into the culture medium may cause a non-cell mediated conversion and subsequently non-specific precipitation of inorganic phosphate mineral, which eventually leads to ectopic or dystrophic mineralization (Terkeltaub, 2001). It is now known that the occurrence of dystrophic mineralization within osteogenic cell culture would impede the further differentiation of progenitor osteoblasts and thus compromise the formation of bone-like extracellular matrix in such culture. However, the mechanism involved is not so universally understood (Parker *et al.*, 2000). In addition, Davies *et al.*, 2002, suggested using the lower concentration of β-GP (5 mM) in murine culture. In far more sensitive human cultures, β-GP was reduced to 3.5 mM and was added only after the appearance of cell multi-layering.

Hydroxyproline is an amino acid that is essential in stabilizing the collagen triple helix. With the absence of this essential amino acid, stable collagen fibers cannot be assembled. In osteogenic medium, ascorbic acid is commonly added to serve as the

cofactor for the hydroxylation of proline. Many studies on bone marrow cultures reported the used of 50 μM ascorbic acid. However, ascorbic acid is relatively unstable, and a half-life of 15.5 hours at room temperature and almost 100 % degradation on the first day of culture at 37 ^{0}C were reported (Vater *et al.*, 2011). Therefore, Parker *et al.*, 2000 recommended that same amount of fresh ascorbic acid need to be added every time when new osteogenic medium is prepared or else long-acting ascorbic acid (ascorbic acid-2-phosphate) should be employed.

According to Jaiswal *et al.*, 1997, when purified MSC are cultivated in the presence of osteogenic supplements, they undergo a developmental cascade defined by the acquisition of cuboidal osteoblastic morphology, transient induction of ALP activity and deposition of a hydroxyapatite-mineralized extracellular matrix. Gene expression studies show that at the late phase of osteogenesis, ALP is transiently increased with concomitant up-regulation of osteopontin (Liu *et al.*, 1997), sialoprotein and osteonectin (Bruder and Chaplan, 2000). However, type I collagen is down-regulated during the late phase of osteogenesis. In addition, a comprehensive series of pulse-chase and transient exposure experiments using dexamethasone to determine which steps of the osteogenesis pathway were dependent on exogenous factor, and which were supported by either paracrine/autocrine factors in culture or sustained lineage progression events following brief exposure to dexamethasone were reported (Bruder and Jaiswal, 1995; Jaiswal and Bruder, 1996). Several investigations showed that dexamethasone induced MSC derived from rat (Hanada *et al.*, 1997; Hong *et al.*, 2009; Oliveira *et al.*, 2009), and human (Oshina *et al.*, 2007; Martins *et al.*, 2010; Anderson *et al.*, 2011), expressed elevated of ALP level. However, mouse MSC undergo osteogenesis with bone morphogenetic proteins (BMPs) but not dexamethasone (Balk *et al.*, 1997).

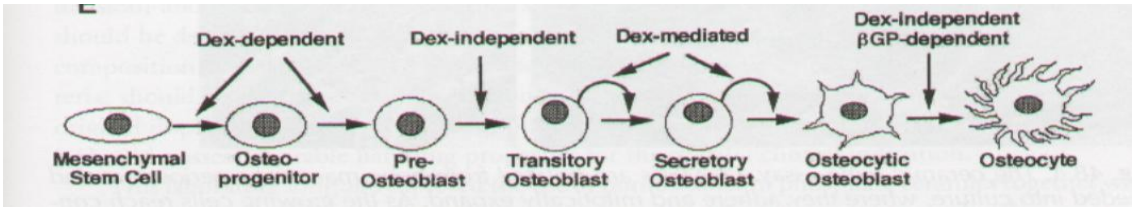


Figure 1.9: Dexamethasone-induced osteogenic differentiation of MSC *in vitro*. (Adopted from Lanza RP, 2000)

There are some simple and common methods employed to characterize and confirm the osteogenicity of culture. The data are then compared with data obtained from already differentiated osteoblasts and non-osteogenic cells. Detection of ALP activity in the culture both by staining technique or colorimetry is commonly associated with osteogenic phenotype and believed to be involved in the initial steps of mineralization of bone extracellular matrix (Hoemann *et al.*, 2009). However, a more complete characterization of osteogenic phenotype of the culture should be carried out. For example, immunohistochemistry and Western blot are conducted to detect bone matrix proteins such as osteopontin, osteocalcin, bone sialoprotein, as well as type I collagen, whereas Von-Kossa staining and alizarin S red staining techniques are used to demonstrate formation of calcium containing minerals (Karmatschek *et al.*, 1997; Quasnichka *et al.*, 2005; Donzelli *et al.*, 2007; Ferron *et al.*, 2010; Hildebrandt *et al.*, 2010; Trentz *et al.*, 2010). All these methods demonstrate the clear sign for osteogenic phenotype in the culture.

1.3 BIOCHEMICAL ENHANCER IN MEDICINAL HERB

1.3.1 Medicinal Herbs

The growing concerns for general health, chronic disease prevention and aging have fueled general interest in phytonutrients, also referred as phytoceuticals which are plant-derived, natural occurring compounds thought to have curative, preventive, or nutritive properties. Medicinal herb is one of the focused fields in phytotherapeutics (Calixto, 2000). There are variety uses of medicinal herbs, which inherited from human ancestors. In the treatment of certain diseases, for example cancer, it is popular that some people try medicinal herbs as alternative treatment should the conventional therapies fail to cure. There have been many reported cases of successfully treatment of cancer patient by using medicinal herbs. Natural products are typically secondary metabolites produced by plants, fungi, bacteria, protozoan, insects and animals in response to external stimuli such as nutritional changes, infection and competition (Strohl, 2000). Apart from serving as a fertile source of cure for numerous diseases, they are also used as a decoction for cancer throughout the world particularly in traditional Chinese medicine, Native American healing and Ayurveda (Smit *et al.*, 1995; Rocha *et al.*, 2001; Cai *et al.*, 2004; Itharat *et al.*, 2004).

Basically, natural products/biochemical enhancers obtained from medicinal herbs are valuable in the treatment of various diseases and illnesses. They are either used as a source of direct therapeutic agents or serve as a raw material base for the elaboration of more complex semi-synthetic chemical compounds. Beside, the chemical structures derived from these substances can be used as models for new synthetic compounds.

In several developing countries, the use of local traditions medicine is still the mainstream of health care. In fact, UNESCO has studied the use of traditional medicine and medicinal plants as normative basis for the maintenance of good health in most developing countries (Hoareau and DaSilva, 1999). Furthermore, an increasing reliance on the use of medicinal plants in the industrialized nations has been traced to the extraction and development of several medications and therapeutic agents from these plants as well as from traditionally used herbal remedies. Moreover, among the nations, herbal remedies have become more popular in the treatment of minor ailments and also on account of the increasing costs of personal health maintenance.

A survey about ethno biology had been carried out by the Ministry of Environment and Forests, Government of India indicates that there are over 8000 species of plants being used by the people of India (Wakdikar and Marg, 2004). On the other hand, together with acupuncture, herbal medicine is considered the most important health care measure in China, where it has been used for over 2500 years. The World Health Organization (WHO) estimates that 80 % of the world population today uses herbal medicine for some aspect of primary health care. Global herb trading market has been rising from 60 billion USD (estimate RM 180 billion) in year 1997 to 200 billion USD (estimate RM 600 billion). The figure is expected to touch 5 trillion USD (estimate RM 15 trillion) (Cheung, 2012)!

In Asian, the practice of traditional medicine is widespread in China, India, Japan, Korea and other countries. About 40 % of the total medicinal consumption is attributed to traditional medicines in China (Hoareau and DaSilva, 1999). In Japan, herbal medicines preparations are more in demand that mainstream pharmaceutical products. This is indicated in Komiya *et al.*, 2011 whereas 72 % percent of physicians in Japan prescribing some traditional Japanese medicine to patient.

Africa is a rich source of medicinal plants as well. The best known species is *Phytolacca dodecandra*. Extracts of the plant, commonly known as *endod*, are used as an effective molluscicide to control schistosomisisi (Esser *et al.*, 2003; Abebe *et al.*, 2005). Other notable examples are like *Catharanthus roseus*, which yield anti-tumor agents such as vinblastine and vincristine (Chu *et al.*, 1996; Datta and Srivastava, 1997); and *Ricinus communis*, which yields the laxative-castor oil. *Harpagophytum procumbens* and *Hibiscus sabdariffa* are processed to be a crude drug for export from Botswana, Lesotho, Namibia and South Africa and from Sudan and Egypt respectively to many other countries. *Pausinystalia yohimbe* which yields *yohimbine* is also processed and exported from Cameroon, Nigeria and Rwanda (Kumar *et al.*, 2011).

American Indians have long been using medicinal plants like *Eupatorium perfoliatum, Podophyllum peltatum* (Mayapple) and *Panax quinquefolium* (ginseng) in USA. These plants have also been found to be possessing therapeutic value such as anti-inflammatory, antiviral and antioxidant properties (Canel *et al.*, 2000; Ng *et al.*, 2004; Hensel *et al.*, 2011). In Central America medicinal plants have been widely used by native populations. United States have spent USD 7.6 billion to import traditional Chinese medicine products from China in year 2011 (Cheung, 2011).

About 1500 species of medicinal and aromatic plants are widely used in Europe countries like Albania, Bulgaria, Croatia, France, Germany, Hungary, Poland, Spain, Turkey, and United Kingdom (Hoareau and DaSilva, 1999). In Project Rubia which was carried out in several Mediterranean countries, 406 out of 985 catalogued species have been identified to have medical use (Gonzalez-Tejero *et al.*, 2008).

The herbs are believed in helping to stimulate the body's own healing process. Laboratory test to observe the particular active components of herb is very difficult since an herb may have many active components that interact with each other. The

effects produced by the whole plants may not be the same as the isolated purified active compounds of the plants (Treasure).

In Malaysia, variety of local herbs is used by different ethnics. The prevalence of using herbal medicines in Malaysia is very high (Aziz and Tey, 2009). Biodiversity in tropical rain forest in Malaysia ensures continuous and sufficient resources of herbs. However, there is a fact that about 70 % of herbal products are imported from China, India and Indonesia. In order to catch up with recent herbal research development and increase share in global herb market, Malaysia have determined to develop local herbal industry. Malaysia government allocates RM 73.1 million to encourage planting of 6 herbs that have been identified as high value herb: Tongkat Ali (*Eurycoma longifolia*), Misai Kucing (Ort*hosiphon stamineus*), Kacip Fatimah (*Labisa pumila*), Mas cotek (*Ficus deltoidea*), Hempedu bumi (*Andrographis paniculata*) and Dukung anak (*Phyllanthus urinaria*) (Cheung, 2012).

1.3.2 Medicinal Herbs in Fracture Treatment

There has been use of medicinal herbs in fracture management in traditional medicine. In Chinese traditional medicine, several herbs are used individually or in an herbal formulation. Traditionally, the herbal plants or formulations are either made in paste or macerated in alcohol (wine) before being used. The rationale of doing so is based on solvent extraction which alcohol extracts working compound from herb. The paste or alcohol is then applied to fracture site for a certain period. Theoretically, the working compound from herb will diffuse and stimulate growth or differentiation of osteoblast at fracture.

Many herbs are used for fracture treatment in tranditional medicine, only a few of them are proven to be effective in promoting osteoblast growth and differentiation *in*

vitro as well as in animal study through proper scientific approach. The research findings are summarized in the table 1.1.

Table 1.1: Summarized research findings of medicinal herbs on bone cell activities.

Herb	Research finding
Drynaria fortune (Naringin)	Inhibit formation of cutured mouse osteoclast (Jeong *et al.*, 2003)
	Possess stimulative effects on proliferation and differentiation of MC3T3-E1 cells (Jeong *et al.*, 2005)
	Increase proliferation and osteogenic differentiation of human bone mesenchymal stem cell (Peng-Zhang *et al.*, 2009)
P.mirifica	Prevent bone loss in orchidectomized rat (Urasopon *et al.*, 2007)
P.radix	Prevent bone loss in castrated male rat (Wang *et al.*, 2005)
Yukmi-jihang-tang (Herbal formulation)	Inhibition of bone resorption in both mouse calvarial osteoblast culture and ovariectomized rat (Jin *et al.*, 2006)

1.3.3 Herbs used in the study

Two medicinal herbs and an herbal formulation were investigated in this study. Traditional Chinese practitioners have claimed that the herbs and the herbal formulation may have some therapeutic effect on bone fracture as well as osteoporosis since they used the herbs and herbal formulation in fracture treatment. Successful case were published in a traditional Chinese herb magazine and both Traditional Chinese practitioner and patient were interviewed and the therapeutic effect of medicinal herbs was claimed (Tung, 2010).

1.3.3.1 *D.quercifolia*

D.quercifolia is one of the candidates. It is a genus of ferns in the family Polypodiaceae. The plant is commonly known as the oak-leaf fern. It can be found in wild in India, Southeast Asia, Malaysia, Indonesia, Philippines, New Guinea, and Australia. It is a large species with deeply pinnatifid foliage fronds. The nest fronds resemble the leaves of oaks. The sori are either scattered or arranged in two regular rows in between the secondary veins. The traditional uses of the herb include treatment of diarrhoea, typhoid, cholera, chronic jaundice, fever, headache, skin diseases and syphilis. This plant is identified to have anti-microbial activity and anti-inflammatory and analgesic properties (Ramesh *et al.*, 2001; Poonam *et al.*, 2005; Anuja *et al.*, 2010). Several constituents were successfully isolated from *D.quercifolia* such as friedelin, epifriedelinol, amyrin, sitosterol, D-glucopyranoside and naringin in the study of Anuja *et al.*, 2010. *D.quercifolia* was chosen in this study because it is under the same family of Gu-Sui-Bu, which has been well-established for bone healing as mentioned earlier.

1.3.3.2 *Justicia gendarussa*

J.gendarussa is another locally found herb that was used in the present study. It was formerly known as *Gendarussa vulgaris* according to taxonomist. *J.gendarussa* is a monotypic genus of the family Acanthaceae. It is a common medicinal herb, appears as a medium sized tree grown in semishade or no shade. The plant is renowned as a traditional cure for many ailments, such as stomach swelling, lunacy, snake-bite, rheumatism, debility, ulcers, sores, dyspepsia, wound healing and as a decoction for worms (Grosvenor *et al.*, 1995). *J.gendarussa* is reported to have inhibition effect on the microbial proliferation of *Staphylococcus aureus* (Grosvenor *et al.*, 1995). Leaf

extacts of *J.gendarussa* is proven to possess anti-inflammatory activity using human red blood cell membrane stabilization method and carrageenan induced paw oedema (Saleem *et al.*, 2011). The use of this medicinal herb in this study is recommended by a traditional Chinese practitioner, Mr Yeoh Shun Tek (63, Lengkok Hijau Green Lane, 11600 Penang, Malaysia), which he uses this herb to treat fracture.

1.3.3.3 Bo-Gu-Cao formulation

An herbal formulation was obtained from traditional Chinese practitioner Mr Yeoh Shun Tak. He claimed that *J.gendarussa* was not usually used alone on fracture but in an herbal formulation with other Chinese herbs. He has learnt the relevant knowledge from his master and makes the herbal formulation on his own (Tung, 2010). Formula of the herbal extract is not disclosed by him, which he can only reveal that *J.gendarussa* is the main constituent in the herbal formulation. The herbal formulation is named Bo-Gu-Cao formulation.

1.4 Research Objectives

As mentioned earlier, bone fracture is a very common health problem. Worldwide projections estimate that the number of hip fractures by 2050 could range between 7.3 and 21.3 million, which could cost of 100 billion euros in treatment and management of the problem (estimated RM 400 billion) (Johnell, 1997). So far, there is no perfect treatment for bone fracture. A treatment for bone fracture which is more effective and shortens recovery time is still in search of. Since the recovery of bone fracture is much relied on osteoblast regeneration from bone marrow, an effective

enhancer for osteoblast growth is much needed. The present study was designed with the following objectives:

1. To evaluate yield efficiency of each solvent extraction on the medicinal plants.
2. To isolate and purify stromal cells from rat bone marrow.
3. To evaluate the effect of biochemical enhancer obtained from medicinal plant on rat BMSC derived osteoblast growth.
4. To evaluate the effect of biochemical enhancer obtained from medicinal plant on osteogenic differentiation of rat BMSC osteoblast.
5. To determine dose-dependent effect of effective biochemical enhancers on proliferation and osteogenic markers of rat BMSC osteoblast.

CHAPTER 2:

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Equipments/ Instruments

Autoclave (*Labtech*)

Benchtop autoclave (*Omega, Prestige Medical*)

Biological safety cabinet (*SAFEMATE1.2, Bioair*)

Centrifuge (*EBA21, Hettich*)

CO_2 incubator (*Galaxy 170R, New Brunswick*)

Convection oven (*Memmert*)

Electronic balance (*Shimadu*)

Haemocytometer (*Hausser Scientific*)

Freeze-drier (*Freezone 2.5, Labconco*)

Inverted microscope (*Nikon Eclipse TS 100*)

Liquid nitrogen tank (*MVE Millenium 2000 SC20*)

Magnetic stirrer (*MSH-10, Wisestir*)

Microcentrifuge (*1730MR, Gyrozen*)

Microplate reader (*Fluostar Optima, BMG Laltech*)

Multi-channel pipettor (10-200 µl)

Pipettor (10-100 µl, 200-1000 µl)

Rotary evaporator (*N-1000, Eyela*)

2.1.2 Chemicals and Consumables

(A) <u>General</u>

15 ml and 50 ml centrifuge tubes (*TPP*)

Media bottles (100 ml, 250 ml, 1 litre, 2 litres)

PBS tablet, pH 7.3 (*Sigma*)

Sterile blue and yellow pipette tips

Sterile needle, *22 G (0.7 x 38 mm) (Becton Dickinson)*

Sterile syringe, 1 ml, 5 ml, 10 ml and 50 ml *(Terumo)*

Volumetric flasks

(B) Extraction of the plant

Ethanol, *95 % (Acros Organic)*

Hexane, *analytical reagent grade (Acros Organic)*

Ethyl acetate, *analytical reagent grade (Fisher Scientific)*

Diethyl ether, *analytical reagent grade (Fisher Scientific)*

Sodium chloride, *analytical reagent grade (Fisher Scientific)*

Filter paper, *qualitative QL100 (Fisher Scientific)*

(C) Cell culture

96-well and 24-well microplate (*TPP*)

Accutase (*Innovative Cell Technologies*)

Antibiotic-antimycotic solution (*Cellgro*)

β-Glycerophosphoric acid disodium salt, pentahydrate, *98 % (Acros Organics)*

Bottle top filter (*TPP*)

Cryovials (*TPP*)

Culture flask (25 cm^3, 75 cm^3) (*TPP*)

Dexamethasone *(Acros Organics)*

Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (*Sigma*)

DMSO (*Fluka*)

Fetal calf serum (FCS) (*Sigma*)

Filtration unit to sterilize culture medium (*TPP*)

L-Ascorbic acid (Vit. C) *(Duchefa Biochemie)*

L(+)-Glutamine, *99 % (Acros Organics)*

Liquid nitrogen (*MOX Linde*)

Sterile syringe filter (0.22 μm and 0.45 μ) (*TPP*)

2.1.3 Analytical Reagents

Acetic acid (*Analar*)

Alizarin Red S powder (*Sigma, A5533*)

Cell Counting Kit – 8 (*Sigma, 96992*)

Ethanol absolute (*Sigma, 3221*)

Fast green FCF (*Sigma, F7252*)

Formalin (*Sigma, HT501*)

Oil Red O solution (*Sigma, O1391*)

Safranin O (*Sigma, S2255*)

SIGMA*FAST*™ p-Nitrophenyl phosphate Tablets *(Sigma, N2770)*

StemPro® Adipogenesis Kit (*Gibco*)

StemPro® Chondrogenesis Kit (*Gibco*)

StemPro® Osteogenesis Kit (*Gibco*)

2.2 SOLVENT EXTRACTION OF MEDICINAL HERBS

Solvent extraction method was used to obtain compounds of different polarity from medicinal plant. Theoretically, compounds of particular polarity were dissolved in respective solvent after soaking the plant with solvent for a certain time period. The plant had to be ground into powder before soaking process to increase surface over volume ratio. This would increase the speed of dissolving process. The extraction method was referred to Lai *et al.*, 2010 with some modifications. Two medicinal plants which are *D.quercifolia* and *Gendarusssa vulgaris* (Figure 2.1) were used in this study. An herbal formulation containing *J.gendarussa* as the main constituent was also investigated in this study, namely Bo-Gu-Cao formulation. The plants and the formulation powder were provided by herbalist Mr Lim Kok Hong from Gunung Ledang Resort, Tangkak, Johor. *D.quercifolia* and *J.gendarussa* were identified by Dr Sugumaran A/L Manickam of the Institute of Biological Sciences, Faculty of Science, University of Malaya, and voucher specimens were deposited in the herbarium of the Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia, with voucher numbers of KLU15725 (*D.quercifolia*) and KLU47737 (*J.gendarussa*). Summary steps for solvent extraction on medicinal plant are illustrated in Figure 2.2.

(a) **(b)**

Figure 2.1: Two medicinal herbs were investigated in this study. (a) *D.quercifolia*, (b) *Justicia gendarussa*.

Procedures:

a) The plant was cleaned thoroughly to remove dirt and sand using tap water.

b) The cleaned plant was dried under sunlight or in oven at 40 ℃.

c) The completely dried plant was weighed and ground into powder form.

d) The plant powder was macerated with 95 % ethanol at the ratio of approximately 1kg: 1L for three days (Bo-Gu-Cao formulation powder was macerated with 95 % ethanol as received because the formulation was prepared in dry powder by the herbalist).

e) Sodium sulphate was added to the mixture water content before the mixture was filtered with filter paper to separate solvent from undissolved material. (The undissolved material might be macerated with 95 % ethanol again and repeated the step (d) and (e) depending on the colour of the filtrate. The step (d) and (e) would not be repeated once the colour of filtrate was light.)

f) The ethanol in the filtrate was evaporated by rotary evaporator at 30-35 ℃ and the leftover was called ethanolic extract.

g) Ethanolic extract was added with 200ml hexane and soaked for three days.

h) The ethanolic extract-hexane mixture was filtered by filter paper. Hexane in the filtrate was evaporated by rotary evaporator to obtain hexane extract.

i) Residue of filtration of ethanolic extract-hexane mixture was added with water and ethyl acetate at the ratio of 1:1 to perform liquid-liquid extraction.

j) After three days, the extraction was separated using a funnel flask. Water layer was freeze dried to obtain water extract and ethyl acetate layer was evaporated using rotary evaporator.

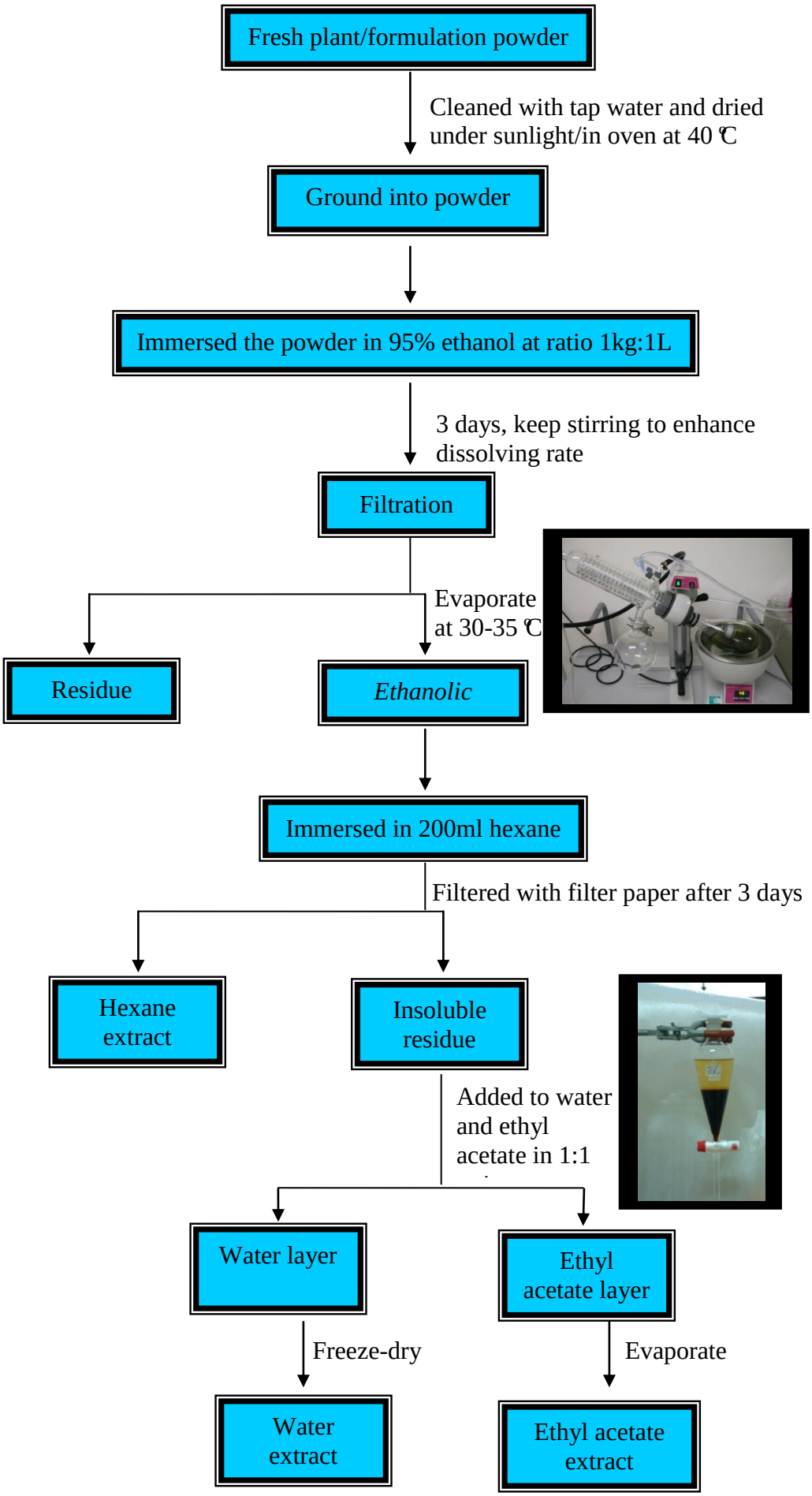


Figure 2.2: Schematic diagram of solvent extraction on medicinal plant.

2.3 RAT BONE MARROW STEM CELLS (rBMSC) ISOLATION AND CULTURE

rBMSC are good source of osteoprogenitor cells. rBMSC can differentiate into BMS derived osteoblasts *in vitro* induced by suitable growth factors, such as dexamethasone, β-glycerophosphate and L-ascorbic acid (Polisetti *et al.*, 2010). In this study, rBMSC were obtained from young adult (5-6 weeks old, male, 150-170 g) Sprague Dawley rats supplied by Animal house, Faculty of Medicine, University of Malaya. Young rats were used instead of adult rats which thought to have more stromal cell obtained due to body size because proliferation and differentiation potential of mesenchymal cells tend to reduce with age (Stolzing and Scutt, 2006; Alt *et al.*, 2012). Prior to experiment, the rats were housed in plastic cages and fed *ad libitum*. The rBMSC isolation protocols were approved by Animal Ethics Committee (Ethics number: BE/16/04/2008/WAB(R)), Faculty of Medicine, University of Malaya (Appendix 1).

2.3.1 Preparations of Primary medium

In this study, primary medium was used to culture rBMSC after isolation from rat. Primary medium is the basic medium that contains fetal calf serum and antibiotics solution. Primary medium used in this study was Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) containing 10 % fetal calf serum (FCS) and 1 % of antibiotic-antimycotic solution (100 units/ml penicillin, 100 µg/ml streptomycin, 25 ng/ml amphotericin).

Procedures:

a) Inside the laminar hood, one bottle of DMEM/F12 powder (D8900, Sigma) was dissolved in 1 liter of sterile, autoclaved double distilled water.

b) 1.2 g of $NaHCO_3$ salt were added to DMEM/F12 solution as a buffer for the media incubated in CO_2 environment.

c) The solution containing DMEM/F12 powder and $NaHCO_3$ was then sterile-filtered by using a bottle-top filter system that was connected to a vacuum pump into a sterile screw-cap bottle, and the resulting solution is called the "sterile DMEM/F12 stock solution".

d) To every 100 ml of DMEM/F12 stock solution, 10 ml of FCS and 1 ml of antibiotic-antimycotic solution were added. The solution was mixed well, sealed with parafilm and kept in refrigerator for future use.

(Note: Properly prepared DMEM/F12 stock solution can be used to prepare primary or complete medium for up to several months when it is stored in refrigerator. However, before use, for every liter of DMEM/F12 stock solution, 0.585 g of L-glutamine was added to replenish the deteriorated L-glutamine originally contained in the medium.)

2.3.2 rBMSC Isolation & Culture

rBMSC isolation and culture protocols described by Maniatopoulos, 1988, were employed for this study with some minor modifications.

Procedures:

(a) Following spinal dislocation and immersion of whole animal in 70 % alcohol solution for 5 minutes, femurs and tibias of SD rats were aseptically excised inside laminar hood.

(b) Soft tissue attached to the excised bones were cleaned off and washed in DMEM/F12 containing 1000 units/ml penicillin and 1000 units/ml streptomycin.

(c) The metaphyseal ends of each excised bones were then cut off and the marrow from the midshaft was flushed with 5ml of primary media (DMEM/F12 containing 10 % fetal calf serum (FCS) and 1 % antibiotic – antimycotic solution)

using a syringe equipped with a 22-gauge needle and collected in a sterile Petri dish.

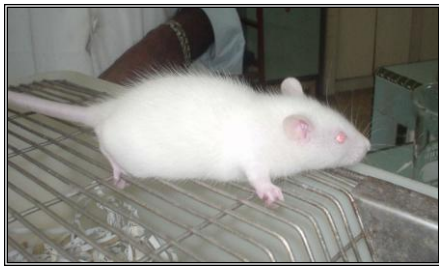
(d) The marrow cell clumps were broken by repeatedly pipetting the cell suspension.

(e) The cells were then collected in a 15 ml sterile test tube and centrifuged at 1500 rpm for 5 min.

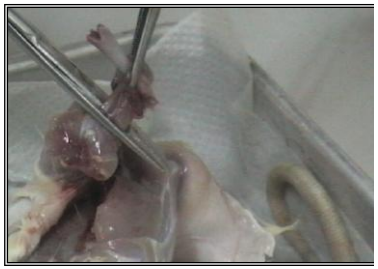
(f) The resulting cell pellets were resuspended in 12 ml of primary media and plated in T-75 flasks (cells from two femurs per flask).

(g) Finally, the plated flasks were incubated in a CO_2 incubator under 5 % CO_2 atmosphere, at 37 ^{o}C and relative humidity of 95 %, for 4 days.

(h) After 4 days incubation in conditioned CO_2 incubator, old medium was discarded together with floating cells. Adherent cell attached to bottom surface would remain and the culture flask was replaced with new primary medium. The steps are diagrammatized in Figure 2.3.



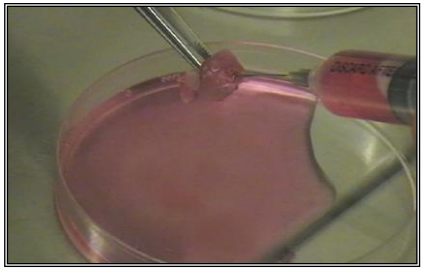
Source: SD rat
(5-6 weeks old)



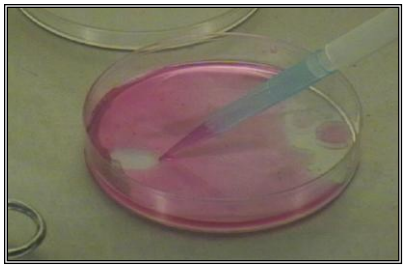
Excision of Femur/Tibia

Washed in primary media containing relatively high concentration of antibiotics

Cutting of metaphyseal ends of femur/tibia



Flushing of bone marrow from midshaft of femur/tibia to sterile petri dish



Breaking of cell clumps by repeated pipetting

Transfer cell suspension to tube for centrifugation at 1500 rpm, 5 minutes

Resuspend cell pellet by repeated pipetting

Plating of cells suspension into T-75 cm^2 flask

Incubation under 5% CO2, at 37 ^{0}C, relative humidity 95%

Figure 2.3: Rat Bone Marrow Stromal Cells (rBMSC) isolation & culture.

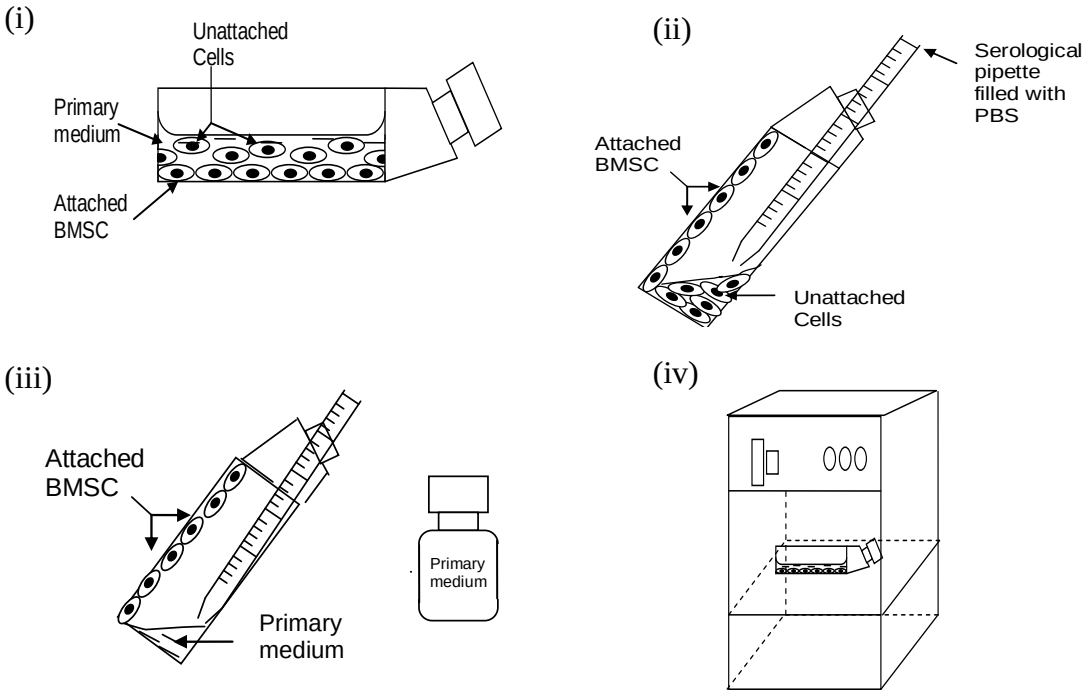


Figure 2.4: BMS-derived osteoblasts culture. (i) BMSC culture in T-75 cm^2 flask, (ii) Removal of unattached cells with PBS, (iii) Addition of fresh primary medium, (iv) incubation under 5% CO_2 atmosphere, at 37^oC and relative humidity of 95% till confluent.

2.3.3 Characterization of rBMSC

Bone marrow is the soft, flexible, vascular tissue found in the hollow interior cavities and cancellous bone spaces in the center of many bones. Hematopoeisis occurs within the bone marrow where the place produces red blood cell, white blood cells and myelocytes. Apart from that, a complex stroma comprising a heterogeneous population of non-haemopoietic cells including fibroblasts, adipocytes, osteoblasts and other cellular elements of bone reside in bone marrow as well. When bone marrow were flushed out from cut femoral and tibia bone, all type of cells mentioned above would be collected in flushed medium. Adherent cells would attach to bottom surface of culture flask. Floating cells were discarded during discarding of medium. The adherent cells are principally mesenchymal

stromal cells. However, bone marrow may contain or can be contaminated by fibroblasts, adipocytes, osteoblasts and other adherent cells during being flushed from long bone, and the unwanted adherent cells could possibly be expanded together with MSC. Thus it is important to characterize BMSC after culture of bone marrow cell is established in laboratory before proceeding to further investigation on rBMSC (Polisetti *et al.*, 2010).

Characteristics of BMSC which distinct BMSC from other cells in bone marrow were identified as below:

a) Fibroblastic appearance of rBMSC: The cells obtained from bone marrow were observed under inverted microscope (Eclipse TS100, Nikon) and images were photographed using NIS Elements BR 3.0 software.

b) Attachment to plastic surface: Adherent of the cells to plastic surface of culture flask was observed using inverted microscope and images were photographed using NIS Elements BR 3.0 software.

c) Mesenchymal stem cells markers confirmation: Flow cytometry was employed to detect mesenchymal stem cells markers on rBMSC used in present study. Mouse anti rat antibodies were used with its fluorescent probes CD31-PE, CD45-FITC, and CD90-PerCP respectively. rBMSC at passage 2 were used and were expected to yield negative for CD31 and CD45 whereas positive for CD90 markers. The procedure are shown as below:

Sample Preparation:

1) Newly isolated BMSC were incubated (5 % CO_2, 37 $^{\circ}$C) for up to Passage 1.

2) The cells were trypsinized at >80 % confluent and then were neutralized with 5ml medium and filtered the samples with cell strainer of mesh size 70 microns followed by 40 microns.
3) Centrifuged for 5 minutes at 1000 rpm and re-suspended the pellet with excess 1X PBS to adjust the final concentration of approximately 10 million cells/ml. Preferably 4mL of cell suspension were prepared.
4) One sample with all 3-color combos and another three control samples that had single color each were prepared according to the steps below:

Staining (Direct and Live Method)

5) Blocked 3-color combo sample by incubating the cell suspension with all three κ-isotype control IgGs (FITC, PE and PerCP) by 1 µg each per 1mL of cell suspension for 10 minutes and did not rinse. Labeled the tube as 3-color combo sample.
6) Blocked each of single color control tube by incubating the cell suspension with its κ-isotype control by 1 µg per 1mL of cell suspension for 10 minutes and did not rinse. Labeled the tube of FITC / PE / PerCP respectively.
7) Another 4 tubes were prepared, and 20 µL of the fluorochrome-conjugated antibodies were added, separately on each three tubes and all 3 antibodies on one tube.
8) One hundred microliter of the prepared cell suspension (equal to 1 million cells) was added to each tube according to its isotype control. At this point, 4 samples were prepared and summarized as follows:

Sample	**Isotype Control**	**Fluorochrome-Conjugated Antibodies**
Test Sample	FITC + PE + PerCP	FITC + PE + PerCP
FITC Control	FITC	FITC
PE Control	PE	PE
PerCP Control	PerCP	PerCP

9) The tubes were vortexed and incubated for 15-30 minutes in a covered ice bucket.

10) 1.5 – 2mL of 1X PBS were added to each tube to wash off excess antibody.

11) Centrifuged in tabletop microfuge for 5 minutes at 2000 rpm.

12) Supernatant was aspirated carefully without disturbing the pellet.

13) Pellets were re-suspended in 500 µL of 1 % paraformaldehyde and transferred into RBT polystyrene tubes.

14) Samples were ready to be acquired within 24 hours exposed to light.

Running the Test

15) The cytometer and computer were started up.

16) BD FACSDiva software was started.

17) Fluid levels in the cytometer window were checked and tanks were prepared as needed.

18) The flow cell was checked for air bubbles.

19) The detectors were verified and optical filters were set according to the specs below:

Blue Laser (488NM solid state) for all runs:		
FITC	(Ex488nm, Em530max) Bluish Green	Bandpass filter: 530/30
PE	(Ex488nm, Em578max) Light Green	Bandpass filter: 585/42
PerCP	(Ex488nm, Em678max) Orange	Bandpass filter:

20) Wait till the laser warm up had finished.

21) The sample was installed on the injection needle.

22) The flow-rate was set in between 10 – 120 µl/min.

23) Clicked Run Sample. All 4 samples ran before moving on to the next step.

Data Analysis

24) The data was collected in voltage output by the software. Then the voltage output was translated either of its height or area to obtain the cell distribution, not width. This distribution can be viewed in all four samples or separately. The software can also isolate and observe dual population relationship, according to the need.

25) The dot plot data of side scatter vs. forward scatter for all four samples was acquired. This shows the distribution of cells according to the complexity vs. size.

26) GATING: From the dot plot, draw a box to gate the region of interest, excluding cell debris and any unwanted distribution. In case of BMSC, the cells may range from 10 to 40 microns. Do this for all 4 samples.

27) SCALING: Next, at Cell count vs. Fluorescent histogram; ensure the curve is fixed to log scale to get a proper fit of both the negative and positive population.

28) COMPENSATION: Using the data from the single color controls, draw two color dot plots of each color combinations and compensate the following pairs:

i. FITC-%PE	ii. PE-%FITC	iii. PerCP-%FITC
iv. FITC-%PerCP	v. PE-%PerCP	vi. PerCP-%PE

Set the data plot on bioexponential scaling before compensating, just in case if there is a value that may pile up on the axis which may lead to error.

29) After compensation, go to Side scatter vs Forward scatter plot of CD90.

30) The 10 to 40 micron region was gated and a log scale histogram was created from it. This should reveal the positive and negative peaks of CD90. As it is hypothesized that CD90 is positive, there should not be any peak on negative side.

31) To look at CD45 and CD31 antigens within CD90 positive, gated the CD90 positive peak, and created a dot plot of CD45 x CD 31 from it.

32) The CD45 x CD31 dot plot can be divided into 4 distinct quadrants. These quadrants should translate as follows:

Quadrant I: CD45 (+); CD 31 (-)

Quadrant II: CD45 (+); CD31 (+)

Quadrant III: CD45 (-); CD31 (-)

Quadrant IV: CD45 (-); CD31 (+)

As it is hypothesized that CD31 and CD45 are negative, no cell or little populations are expected on Quadrant I, II and IV, with most of the cells sitting on Quadrant III.

d) Multilineages differentiation: BMSC are known to be multipotent. BMSC are able to differentiate into several specific mature cells under certain growth factors. Induction of BMSC differentiation into osteoblast, adipocytes and chondrocytes can to prove the multipotent ability of BMSC. StemPro® Adipogenesis Kit (A1007001, Gibco), StemPro® Chondrogenesis Kit (A1007101, Gibco) and StemPro® Osteogenesis Kit (A1007201, Gibco) were used to induce adipogenesis, chondrogenesis and osteogenesis respectively.

Adipogenesis:

1) Primary BMSC were isolated and expanded with primary medium.
2) BMSC were passaged when cultures reach 60 – 80 % confluency. BMSC of passage between 2 to 6 were used in adipogenesis assay.
3) rBMSC were harvested and seeded in 6-well plate at cell density of 3×10^3 to 5×10^5 viable cells/cm^2.
4) rBMSC were expanded in primary medium for 2 to 4 days (to near or complete confluency) before referred with Adipogenesis Differentiation Medium.

5) Oil Red O staining was performed on BMSC after incubated with Adipogenesis Differentiation Medium for 21 days. The procedures for Oil Red O Staining are as below:

   i. 0.5 % Oil Red O solution was prepared by the ratio of 0.5 g Oil Red O in 100 ml propylene glycol.
   ii. The medium was aspirated carefully from well.
   iii. The cells were fixed by 10 % formalin for 5-10 minutes followed by rinsing immediately in 3 changes of distilled water.
   iv. The wells were let air dry for a few minutes.
   v. The cells were soaked in absolute propylene glycol for 2-5 minutes to avoid carrying water into Oil Red O.
   vi. The cells were stained in pre-warmed Oil Red O solution for 8-10 minutes in 60 ℃ oven.
   vii. Differentiated in 85 % propylene glycol solution for 2-5 minutes.
   viii. Rinsed in 2 changes of distilled water.
   ix. The cells were observed under inverted microscope.

Chondrogenesis:

1) Primary rBMSC were isolated and expanded with primary medium.
2) rBMSC were passaged when cultures reach 60 – 80 % confluency. rBMSC of passage between 2 to 6 were used in chondrogenesis assay.
3) rBMSC were harvested and seeded in 6-well plate at cell density of 100 viable cells/5µl medium droplet for 20 droplets per well.
4) rBMSC were let to attach to culture surface in CO_2 incubator for 2 hours before adding with Chondrogenesis Differentiation Medium.

5) Safranin O staining was performed on rBMSC after incubated with Chondrogenesis Differentiation Medium for 14 – 21 days. The procedures for Safranin O Staining are as below:

Reagents:

i. 1.5 % aquos safranin
ii. 0.02 % alcoholic fast green (95 % ethanol)
iii. 1 % acetic acid

Procedures:

i. The medium was aspirated carefully from well.
ii. The cells were rinsed with distilled PBS.
iii. The cells were fixed by 10 % Formalin for 5 – 10 minutes followed by rinsing with water.
iv. The fixed cells were put in 1.5 % Safranin O for 40 minutes and followed by rinsing in distilled water for 3 times.
v. The well was flooded with 0.02 % alcoholic fast green for 30 seconds followed by 1 % acetic acid 3 seconds.
vi. Then the well was rinsed quickly with distilled water.
vii. The well was flooded with 95 % ethanol for 1 minute.
viii. The cells were dehydrated in 2 changes of 100 % ethanol with 1 minute each.
ix. The cells were observed under inverted microscope.

Osteogenesis:

1) Primary rBMSC were isolated and expanded with primary medium.
2) rBMSC were passaged when cultures reach 60 – 80 % confluency. BMSC of passage between 2 to 6 were used in osteogenesis assay.

3) rBMSC were harvested and seeded in 6-well plate at cell density of 3 x 10^3 to 5 x 10^5 viable cells/cm^2.
4) rBMSC were expanded in primary medium for 2 to 4 days (to near or complete confluency) before referred with Osteogenesis Differentiation Medium.
5) Alizarin Red S staining was performed on BMSC after incubated with Osteogenesis Differentiation Medium for 21 days. The procedures for Alizarin Red S Staining are as below:
    i. Alizarin Red S Solution was prepared by disolving 2g Alizarin Red S powder in 100ml distilled water.
    ii. The medium was aspirated carefully from well.
    iii. The cells were fixed by incubating in iced cold 70 % ethanol for 1 hour at room temperature.
    iv. The alcohol was aspirated carefully and rinsed twice (5 – 10 minutes each) with water.
    v. The water was aspirated and excess Alizarin Red Solution was added to cover the wells.
    vi. The well plate was incubated at room temperature for 30 minutes.
    vii. After 30 minutes, Alizarin Red Solution was removed and the wells were washed 4 times with 1 ml water and aspirated after each wash.
    viii. One to one and half mililiter water were added to each well to prevent the cells from dying.
    ix. The cells were observed under inverted microscope.

2.3.4 rBMS Derived Osteoblasts Culture

rBMSC could be chemically-induced to form osteoblasts by culturing the cells in osteogenic medium (Parker E *et al*., 2000). Basically, osteogenic medium is a primary medium supplemented with factors that can induce BMSC to differentiate into osteoblasts. These factors are dexamethasone (Canalis E (1985), Maniatopoulos C *et al.* (1988)), β-GP (Tenenbaum HC *et al.*, 1989), and L-ascorbic acid (Hosseini MM *et al.*, 1996).

2.3.4.1 Preparation of Osteogenic Medium (OM).

Materials:

i. DMEM/F12 primary medium
ii. Dexamethasone (10 nM)
iii. β-GP (5 mM)
iv. L-ascorbic acid (50 μg/ml)

Calculations:

(All of the following stock solutions were calculated to prepare 100 ml of osteogenic or complete medium.)

(a) For 10 nM dexamethasone, a 10 μM dexamethasone stock solution was prepared. First, 0.0039 g of dexamethasone was dissolved in 100 ml of double-distilled water. Then, 1 ml of the solution was added into 99 ml of double-distilled water. Finally, the second solution was sterile-filtered using syringe filter, collected in a screw-cap bottle and kept in the refrigerator for future use.

10 nM Dexamethasone

= 1×10^{-8} M (m.w = 392.46)

= (1×10^{-8} mol/L) (392.46)

= 3.9246×10^{-6} g/L

= 3.9246×10^{-7} g/100 ml of complete medium

(b) For 5 mM β-GP, a 50 mM β-GP stock solution was prepared. Basically, 1.5306 g of β-Glycerophosphoric acid disodium salt was dissolved in 10 ml of double-distilled water. The solution was then sterile-filtered by using syringe filter, collected in a sterile 10 ml centrifuge tube and kept in the refrigerator for future use.

5 mM β-GP = (5×10^{-3} mol/L) (306.11) (m.w = 306.11)

= 1.5306 g/L

= 0.153 g/100 ml of complete medium

(c) 50 μg/ml of L-ascorbic acid was prepared fresh every time when preparing new complete medium. Basically, 0.005 g of L-ascorbic acid was dissolved in 1 ml of double-distilled water, followed by sterile-filtration using a syringe filter.

Procedures:

1) 10 μM Dexamethasone and 50 mM β-GP stock solution were prepared as described above.
2) Inside the laminar hood, 1 ml of Dexamethasone and β-GP stock solution, respectively, were added into a sterile 100 ml screw-cap bottle. This is followed by the addition of 1ml of the freshly prepared L-ascorbic acid.
3) Approximately 100 ml of DMEM/F12 primary medium (supplemented with 10 % fetal calf serum, and 100 units/ml penicillin and 100 units/ml streptomycin) were then added

into the bottle. The medium solution was mixed well and kept in refrigerator for future use.

2.3.4.2 rBMS Derived Osteoblasts Culture

rBMSC obtained were chemically induced to differentiate into osteoblastic cells by incubating in OM in a CO_2 incubator under 5 % CO_2 atmosphere, at 37 ^{o}C and relative humidity of 95 %.

Procedures:

(a) Inside the laminar hood, old medium in flask plated with BMSC was discarded by using sterile serological pipette.

(b) Then, the flask was rinsed with sterile PBS repeatedly to remove unattached cells, especially the haematopoietic cells.

(c) 12 ml of fresh OM were then added into the flask by using a sterile serological pipette.

(d) Finally, the flask filled with OM was incubated in CO_2 incubator under 5 % CO_2 atmosphere, at 37 ^{o}C and relative humidity of 95 %.

(e) The medium was changed every 2 days and the rate of cell growth was monitored by regular observation under inverted microscope.

2.4 SCREENING OF EFFECT OF BIOCHEMICAL ENHANCER ON rBMS DERIVED OSTEOBLAST

Effect of 12 herbal extracts (*D.quercifolia* ethanolic extract, *D.quercifolia* hexane extract, *D.quercifolia* ethyl acetate extract, *D.quercifolia* water extract, *J.gendarussa* ethanolic extract, *J.gendarussa* hexane extract, *J.gendarussa* ethyl acetate extract, *J.gendarussa* water extract, Bo-Gu-Cao formulation ethanolic extract, Bo-Gu-Cao formulation hexane extract, Bo-Gu-Cao formulation ethyl acetate extract and Bo-Gu-Cao formulation water extract) were tested on rBMS derived osteoblast and compared to control using ANOVA statistic analysis. Proliferation and ALP activity were the main evaluated parameters. Proliferation was reflected by OD in CCK-8 assay and the result was shown in estimation of cell number by referring the OD to standard curve of known cell number. ALP activity was evaluated by colorimetric method as the ALP activity hydrolyzed substrate to a yellow colored end-product, 4-nitrophenol. The OD was converted to conversion of pNPP to p-nitrophenol per minute by referring to 4-nitrophenol standard curve and followed by normalizing to total protein content. The result was express in ALP activity/ quantity of protein content (mol/minute/μg protein).

2.4.1 Preparation of herbal extract reconstituted in medium

One concentration of herbal extract was used in screening of effect herbal extract which was 100 μg/ml in OM. As all 4 types of solvent extract can be dissolved in dimethyl sulphoside (DMSO), the herbal extracts were dissolved in DMSO prior to mixing in OM. The steps of herbal extract preparation are summarized as below:

a) Fifty milligram of the herbal extract was dissolved in 1ml of DMSO, thoroughly.

b) Mixed 0.1ml of 50mg/ml herbal extract with 0.9ml of OM to make 5mg/ml stock solution.

c) The sample was filtered with 0.45 µm disposable syringe filter.

d) The filtrate was collected in sterile microtube.

e) Two hundred microliter of filtered herbal extract in DMSO was mixed with 800 µl OM to make 1 ml of 1mg/ml herbal extract.

f) The 1mg/ml herbal extract was then subjected to proliferation rate evaluation assay, ALP detection and Alizarin Red S staining. Control solution was prepared by adding 200 µl DMSO with 800 µl OM.

2.4.2 Proliferation rate evaluation on herbal extract effect on rBMS derived osteoblast

Proliferation of rBMS derived osteoblast was evaluated using Cell Counting Kit – 8 (CCK-8) purchased from Sigma Aldrich (96992, Sigma). CCK-8 allows very convenient assays by utilizing the highly water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] produces a water-soluble formazan dye upon reduction in the presence of an electron carrier. There is another similar and commonly used assay for proliferation named MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983). However, CCK-8 was chosen over MTT because using of CCK-8 involves lesser steps and cells need not to be sacrificed at the end of the assay. WST-8 in CCK-8 is reduced by dehydrogenases in cells to give a yellow colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. The detection

sensitivity of CCK-8 is higher than other tetrazolium salts such as MTT. Reduction of WST-8 to yellow colored formazan is shown in figure 2.5.

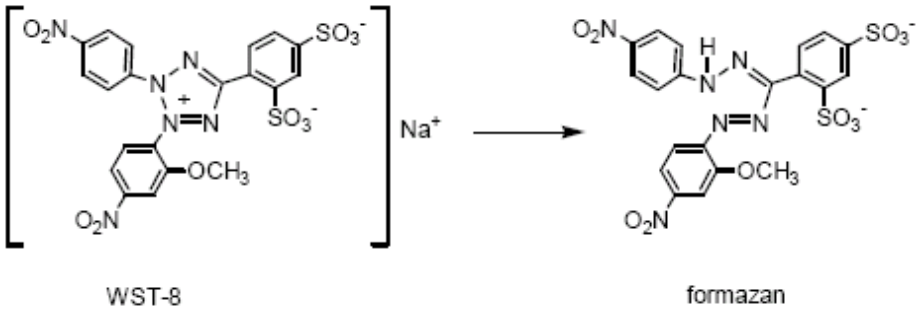


Figure 2.5: Structures of WST-8 and formazan.

2.4.2.1 Generating standard curve of cell number

Standard curve of cell number was generated using CCK-8 on various known cell numbers to assist in determining cell number in proliferation rate of rBMS derived osteoblast after incubation with herbal extract. The procedures are listed as below:

a) rBMS derived osteoblast at passage 2 to 6 was harvested using accutase and seeded in 96-well plate at various density of cell/well: 2 x 10^3 – 2.4 x 10^4 cell/well (The recommended maximum cell seeding number per well is 2.5 x 10^4 according to manual provided by Sigma Aldrich) in 200 µl.

b) The cells were then incubated in CO_2 incubator (95 % humidity, 37 ^oC, 5 % CO_2) for 6 hours.

c) CCK-8 was thawed from freezer in water bath at 37^oC 5 minutes prior to adding CCK-8 solution to 96-well plate.

d) 10 µl of CCK-8 solution was added to each well of the plate. Prior to addition of CCK-8 solution, old medium was discarded and replaced with 100 µl fresh OM.

e) The plate was further incubated for 3 hours in CO_2 incubator.

f) The absorbance was measured at 460 nm using microplate reader (FLUOSTAR OPTIMA, BMG Labtech) and the optical density (OD) obtained was plotted against number of cell.

g) Figure 2.6 shows an diagrammatic example of orientation of cell seeding in 96-well plate for generating standard curve of cell number in this study

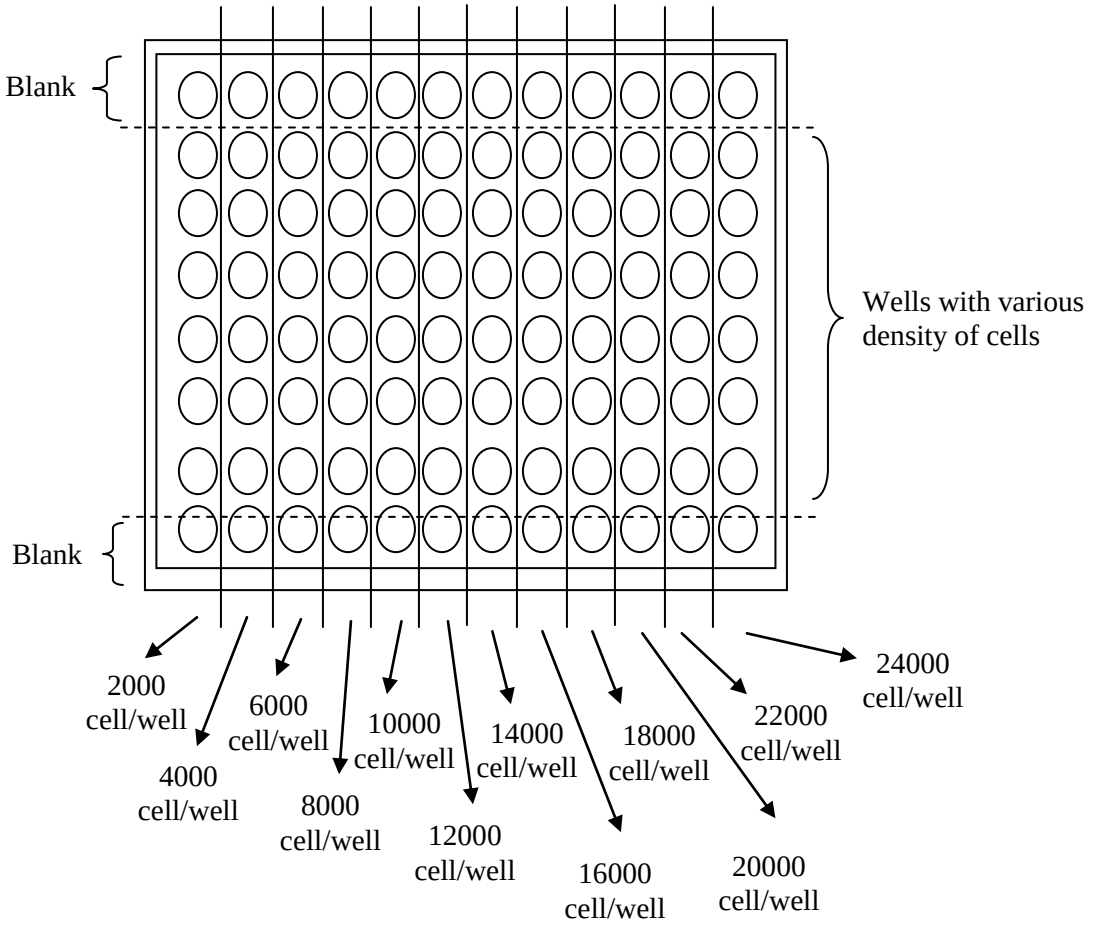


Figure 2.6: Various number cell seeding density in a 96-well plate. $n = 6$.

2.4.2.2 Proliferation rate of rBMS derived osteoblasts after incubation with herbal extracts

The rBMS derived osteoblasts were incubated with 12 types of herbal extract sample prepared in section 2.4.1 for 3 days, 7 days and 14 days in 96-well plate. The initial cell seeding number is 5 x 10^3 cell/well. The proliferation rate was evaluated using CCK-8. The details of the steps are as described below:

a) rBMS derived osteoblasts (passage 2 – 6) were harvested using accutase and seeded at density 5 x 10^3 cell/well in 200 µl OM.

b) The cells were allowed to attach at the bottom surface of well plate after incubating overnight in CO_2 incubator at 95 % humidity, 37 ^oC and 5 % CO_2.

c) Prior to addition of herbal extract after overnight incubation, old medium was discarded and replace with 180 µl OM.

d) Twenty µl of 1mg/ml herbal extract were added to each well. The final concentration of herbal extract in each was 100 µg/ml.

e) Twenty µl of control solution was added to control wells.

f) The 96-well plate was incubated in CO_2 incubator for 3 days, 7 days and 14 days. The cells were refed with new OM and herbal extract (control solution for control wells) for every 2 – 3 days.

g) Proliferation rate evaluation using CCK-8 was carried out after the designated incubation period.

h) The OD was then converted to cell number by comparing to standard curve obtained in section 2.4.2.1 and compared to cell number in control wells.

i) Figure 2.7 shows an example of cell seeding in 96-well plate for proliferation rate assay in this study.

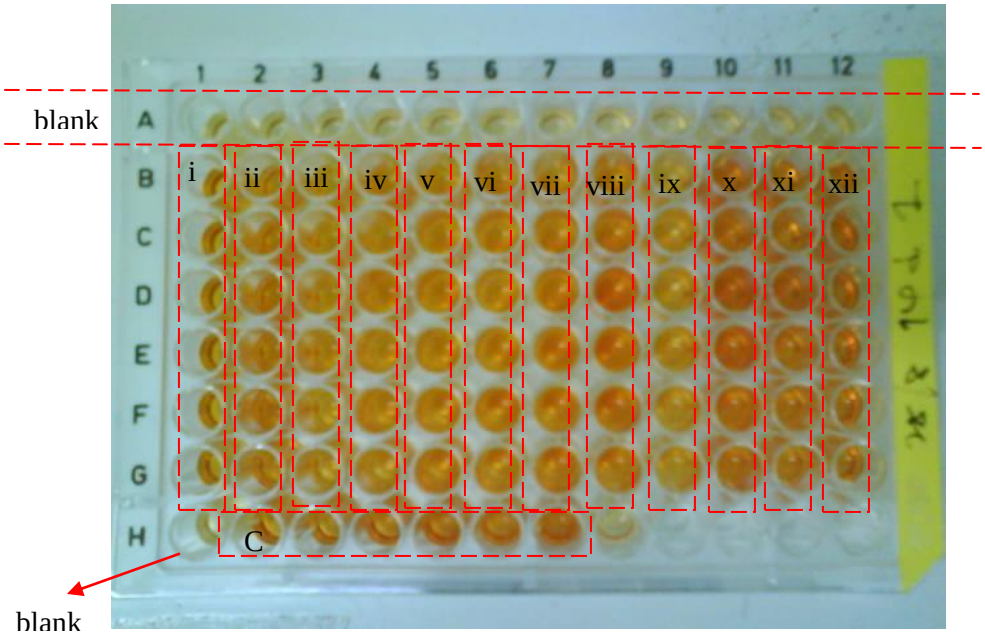


Figure 2.7: An example of 96-well microplate for proliferation rate assay used in the study. Top rows and well H1 were blank (i.e. no cells). 6 wells were seeded with rBMS derived osteoblasts and incubated with OM and herbal extract. i = *D.quercifolia ethanolic extract*, ii = *D.quercifolia* hexane extract, iii = *D.quercifolia* ethyl acetate extract, iv = *D.quercifolia* water extract, v = *J.gendarussa ethanolic extract*, vi = *J.gendarussa* hexane extract, vii = *J.gendarussa* ethyl acetate extract, viii = *J.gendarussa* water extract, ix = Bo-Gu-Cao formulation *ethanolic extract*, x = Bo-Gu-Cao formulation hexane extract, xi = Bo-Gu-Cao formulation ethyl acetate extract, xii = Bo-Gu-Cao formulation water extract and C = control. *n* = 6.

2.4.3 ALP detection assay

ALP is a membrane-bound enzyme, which is commonly associated with the osteogenic phenotype and is believed to be involved in the early steps of mineralization of bone extracellular matrix (Hoemann *et al.*, 2009). The detection of ALP or quantifying the levels of ALP present in the culture indicates the osteogenicity of rBMS derived osteoblasts under influence of herbal extract. Commercially available standard kit - SIGMA*FAST*™ p-Nitrophenyl phosphate Tablets (N2770, Sigma) was used in this study. The SIGMA*FAST*™ p-Nitrophenyl phosphate Tablets consists of p-nitrophenol phosphate (pNPP), buffer and the required magnesium cations. It demonstrates a high sensitivity for the detection of ALP activity as a soluble end-product is produced when pNPP serves as

substrate of choice in ALP enzyme immunoassays. The soluble yellow end-product absorbance is read at 405 nm. The reaction involved is illustrated as below:

$$\text{p-NPP (Colorless)} + H_2O \xrightarrow{\text{ALP}} \text{p-Nitrophenol (Yellow)} + H_3PO_4$$

2.4.3.1 Generating standard curve of 4-nitrophenol

A standard curve was generated prior to ALP detection assay by using OD obtained from various concentration of 4 – nitrophenol (1048, Sigma) in distilled water. p–nitrophenol is the end-product of reaction of ALP on pNPP substrate. By comparing the OD produced by ALP on pNPP to OD of known concentration of 4 – nitrophenol standard curve, quantity of pNPP converted to nitrophenol can be estimated. In another word, activity of enzyme ALP over time is estimated. Triplicates of each concentration of 4 – nitrophenol ranged from 0.002 µmol/ml – 0.020 µmol/ml were prepared in a 96-well plate and read at 405 nm. Standard curve was plotted by using quantity of 4 – nitrophenol versus OD.

2.4.3.2 Obtaining cell lysate for total protein content and ALP activity determination

The sample used in ALP detection assay was cell lysate of rBMS derived osteoblasts after certain incubation period with herbal extract. As mentioned earlier, ALP is a membrane-bound enzyme. The cells were lysed and cell membrane was broken in order to extract ALP by M-PER® Mammalian Protein Extraction Reagent (78501, Thermo Scientific). The reagent is able to extracts cytoplasmic and nuclear protein from cultured mammalian cells. Cell lysis using this reagent is rapid, mild and efficient and the cell lysate is compatible with protein assays. The procedures of cell cultures assay for obtaining cell lysate sample are listed as below:

a) RBMS derived osteoblasts were harvested at passage 2 – 6.

b) The cells were seeded at density of 1 x 10^4 cell/well in a in 24-well plate, with 1 ml OM in each well.

c) The cells in 24-well plate were allowed to attach to bottom surface of microplate by incubating the plate in CO_2 incubator (95 % humidity, 5 % CO_2 and 37 ^oC) overnight.

d) On the second day, old medium was replaced with 0.9 ml fresh OM.

e) Zero point one ml of 1 mg/ml herbal extract was then added to each well. The final concentration of herbal extract in each was 100 µg/ml.

f) The 24-well plate was incubated in CO_2 incubator for 7 days, 14 days and 21 days as the ALP is produced by osteoblast. The cells were refed with new OM and herbal extract (control solution for control wells) for every 2 – 3 days.

g) The cells were harvested and subjected to cell lysis after designated culture period. Briefly, medium of each well was discarded and the wells were rinsed with PBS twice to completely wash off medium. Phenol red and serum in medium could interfere with action of the M-PER® Mammalian Protein Extraction Reagent as well as subsequent protein analysis. This was followed by adding 200 µl of the reagent to each well. The plate was shaken gently for 5 minutes. The cell lysate was collected and transferred to microcentrifuge tube. This was followed by centrifuging the lysate at 13000 rpm for 5 minutes at 4^oC in a temperature controlled microcentrifuge (Gyrozen 1730MR). Temperature was controlled at 4^oC in microcentrifuge to avoid production of heat that could denature protein by high speed rotation. The supernatant was transferred to a new tube and subjected to total protein content and ALP activity determination.

* *Three plates were used in one experiment run as 6 wells in a 24-well plate were treated with one type of herbal extract. Thus,sample size is considered as n = 6.*

2.4.3.3 Total protein content determination

Total protein content in cell lysate was determined prior to ALP detection. The result of ALP detection was express after normalized to total protein content. This would give a better indication of how strong was the osteogenic expression of the cells by determining ALP in total protein. The steps were summarized as below:

a) A protein standard was prepared using bovine serum albumin (P8994, Protein Standard) in buffer ranging from 1 mg/ml – 10 mg/ml in a 96-well plate, with triplicates were prepared for each concentration.
b) Triplicates of samples obtained from section 2.4.3.2 were transferred to the 96-well plate, with 10 µl in each well.
c) Two hundred and fifty µl of the Bradford Reagent (B6916, Sigma) were added to each well and mixed gently by shaking the plate with an shaker for approximately 30 seconds.
d) The plate was incubated at room temperature for 5 – 45 minutes.
e) The absorbance of the reaction mixture was measured at 595nm. (Samples must be measured within 1hr as the protein-dye complex is stable up to 1 hour).
f) Net absorbance versus the protein concentration of each standard was plotted.
g) The protein concentration of the samples was determined by comparing the net A_{595} values against the standard curve.

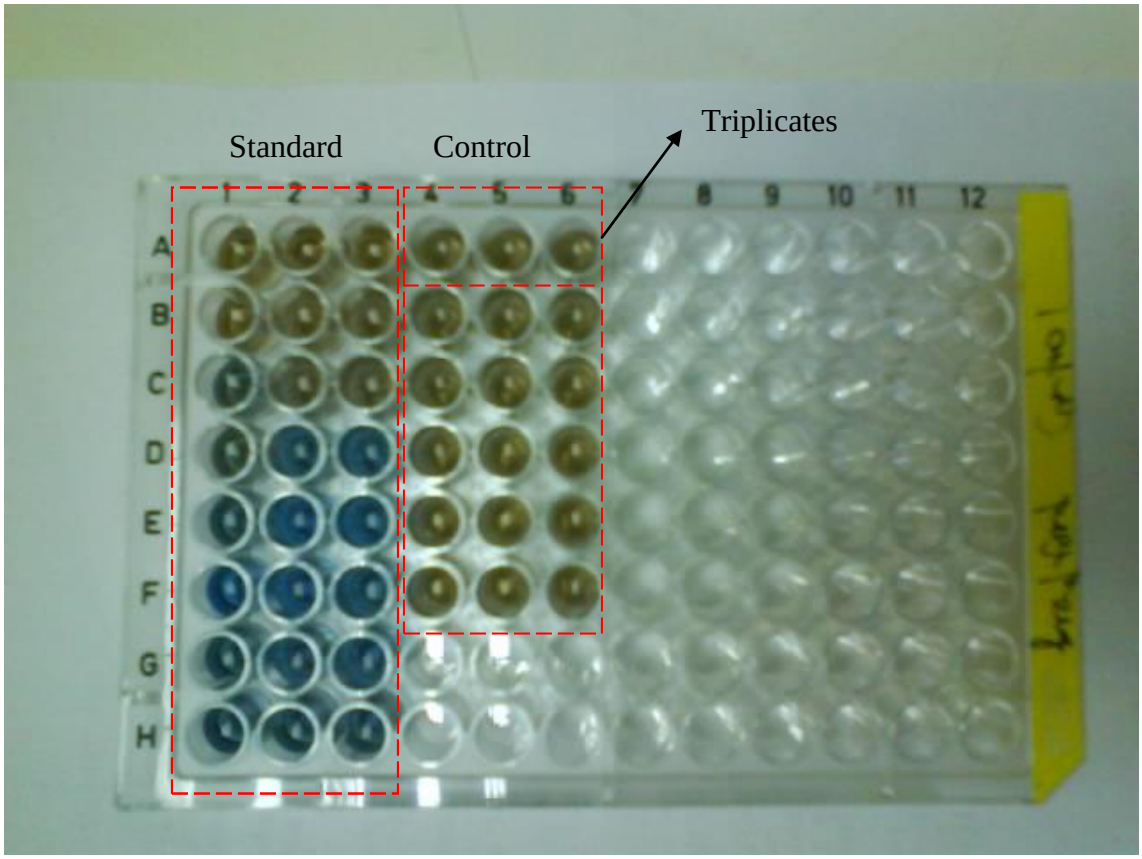


Figure 2.8: Known concentrations of protein standard and samples of control after reaction with Bradford Reagent. Bradford Reagent turned bluer if the protein content was higher. Each sample was prepared into triplicates.

2.4.3.4 ALP activity determination

ALP activity in cell lysate obtained from rBMS derived osteoblasts was determined by ALP detection assay employing protocol provided together with SIGMA*FAST*™ p-Nitrophenyl phosphate Tablets with minor modifications. The steps were summarized as below:

a) PNPP substrate was prepared by dissolving a SIGMA*FAST* p-Nitrophenyl phosphate tablets set (N2770, SIGMA) in 20 ml distilled water.

b) Triplicates of samples with 50 µl each were transferred into 96-well plate.

c) 200 µl of pNPP substrate were added to samples.

d) The plate was incubated in dark for 30 minutes at room temperature.

e) Absorbance of each well was read at 405 nm immediately after 30 minutes incubation.

f) Comparisons were made on the mean absorbance values rBMS derived osteoblast incubated with control solution and herbal extracts, as indications for ALP activity. The result was expressed as ALP activity over time in each µg of protein. Percentage increase of ALP production was also calculated, in order to compare the percentage increase of ALP values for the first (7 to 14 days of incubation) and second (14 to 21 days of incubation) incubation intervals. Calculation of ALP activity over time in each µg of protein are expressed as below:

$$\frac{\text{ALP activity (after referring OD to standard curve)}}{\text{µl (samples volume) x µg protein (quantity of protein)}} = \text{ALP activity/min/µl/µg protein}$$

2.4.4 Alizarin Red S Staining for Calcium Deposition

Calcium deposition or mineralization is a sign of maturation of osteoblast and is also an important indicator of level osteogenic expression of rBMS in this study. Alizarin Red S is an anthraquinone derivative that may be used to identify calcium in fixed tissue layer. Principally, calcium forms an alizarin red S-calcium complex in a chelation process. The reaction is birefringent. The cells containing calcium deposits were stained orange-red by Alizarin Solution. The method used in present study was adopted from study of (Gregory *et al.*, 2004) with modification. Briefly, rBMS derived osteoblast at passage 2 – 6 were harvested and seeded at density of 1 x 10^4 cell/well in 1 ml of OM on a 24-well plate and allowed to attach at the bottom of the plate by incubating the plate in CO_2 incubator

(95 % humidity, 5 % CO_2, 37^{o}C) overnight. This was followed by replacing old medium with fresh medium with herbal extract at which the concentration of herbal extract in fresh medium was 100 µg/ml. The plate was incubated for further 7, 14 and 21 days and medium was changed every 2 – 3 days before proceeded to Alizarin Red S staining as described in the staining protocol in section 2.3.3. Figure 2.11 shows the cells staining with Alizarin Red S solution after 21 days incubation.

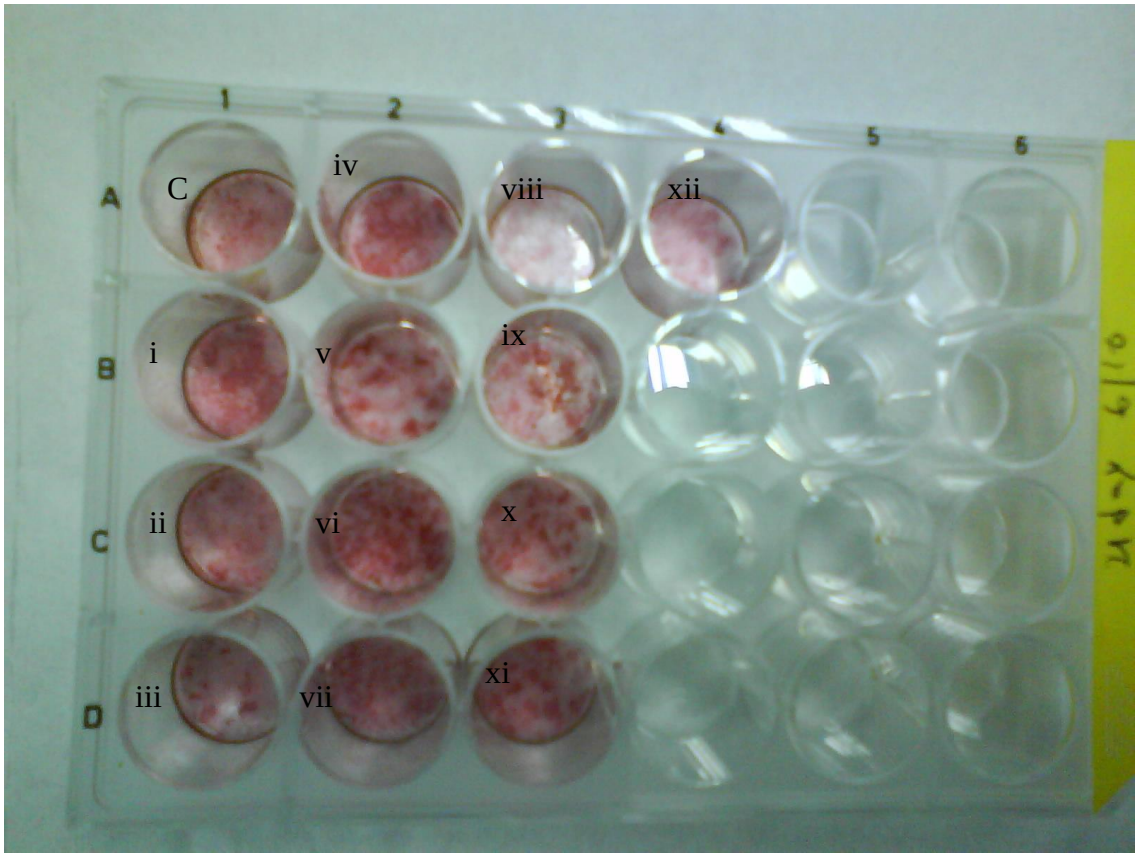


Figure 2.9: RBMS derived osteoblasts after incubation with different herbal extract for 21 days were stained with Alizarin Red S staining. i = *D.quercifolia* ethanolic extract, ii = *D.quercifolia* hexane extract, iii = *D.quercifolia* ethyl acetate extract, iv = *D.quercifolia* water extract, v = *J.gendarussa* ethanolic extract, vi = *J.gendarussa* hexane extract, vii = *J.gendarussa* ethyl acetate extract, viii = *J.gendarussa* water extract, ix = Bo-Gu-Cao formulation ethanolic extract, x = Bo-Gu-Cao formulation hexane extract, xi = Bo-Gu-Cao formulation ethyl acetate extract, xii = Bo-Gu-Cao formulation water extract and C = control.

2.5 DOSE DEPENDENCY EFFECT OF HERBAL EXTRACTS ON RBMS DERIVED OSTEOBLASTS GROWTH AND DIFFERENTIATION

Dosage of herbal extract could play important role in triggering rBMS derived osteoblasts response. Cell may react differently towards different amount of working compound within herbal extract. For an example, proliferation and ALP level of osteoblasts increased significantly when incubated with *Fructus cnidii* at concentration from 40 mg/ml – 320 mg/ml. Lower and higher concentration of the herb stimulated no significant effect on osteoblasts when compared to control (Zhang *et al.*, 2010). Theoretically there is a threshold dosage presented in order to trigger cell response, no cell response when incubating with concentration of herbal extract below threshold dosage. At the same time, the response of rBMS derived osteoblasts towards concentrations higher than threshold dosage (if there was any) was to be examined as well. Water extract *D.quercifolia* and *J.gendarussa* were prepared in various concentrations and to be tested on rBMS derived osteoblasts based on the result obtained from section 2.4.

2.5.1 Preparation of *D.quercifolia* and *J.gendarussa* water extract at various concentrations

Five concentrations of herbal extract is used in screening of effect herbal extract which are 10 µg/ml, 50 µg/ml, 150 µg/ml, 250 µg/ml and 500 µg/ml in OM. Water extracts of two herbs are soluble in OM directly. The preparation of the herbal extracts was just dissolving the herbal extracts in OM. The steps of herbal extract preparation are summarized as below:

a) Fifty milligram of the herbal extract was dissolved in OM, thoroughly.

b) Mixed 0.1ml of 50mg/ml herbal extract with 0.9ml of OM to make 5mg/ml stock solution.

c) The sample was filtered with 0.45 μm disposable syringe filter.

d) The filtrate was collected in sterile microtube.

e) Various amount of stock solution was mixed with OM as the table below to make 1 ml of OM with certain concentration of herbal extract.

Table 2.1: Preparations of various concentrations of herbal extracts reconstituting with OM.

Stock solution (μl)	0	20	100	300	500	1000
OM (μl)	1000	980	900	700	500	0
Concentration (μg/ml)	0	100	500	1500	2500	5000
Final concentration in culture plate (μg/ml)	0	10	50	150	250	500

f) The herbal extracts at different concentrations were then subjected to proliferation rate evaluation assay, ALP detection osteocalcin level evaluation and Alizarin Red S staining. Concentration at 0 μg/ml was considered as control solution.

2.5.2 Proliferation rate of rBMS derived osteoblasts after incubation with various concentration of *D.quercifolia* and *J.gendarussa* water extract

The proliferation rate was evaluated by using CCK-8 which was discussed in section 2.4.2. The incubation of various concentrations of the two extracts was shown in figure 2.10. The initial cell seeding number was 5 x 10^3 cell/well. The steps of the procedures employed were as same as in section 2.4.2.

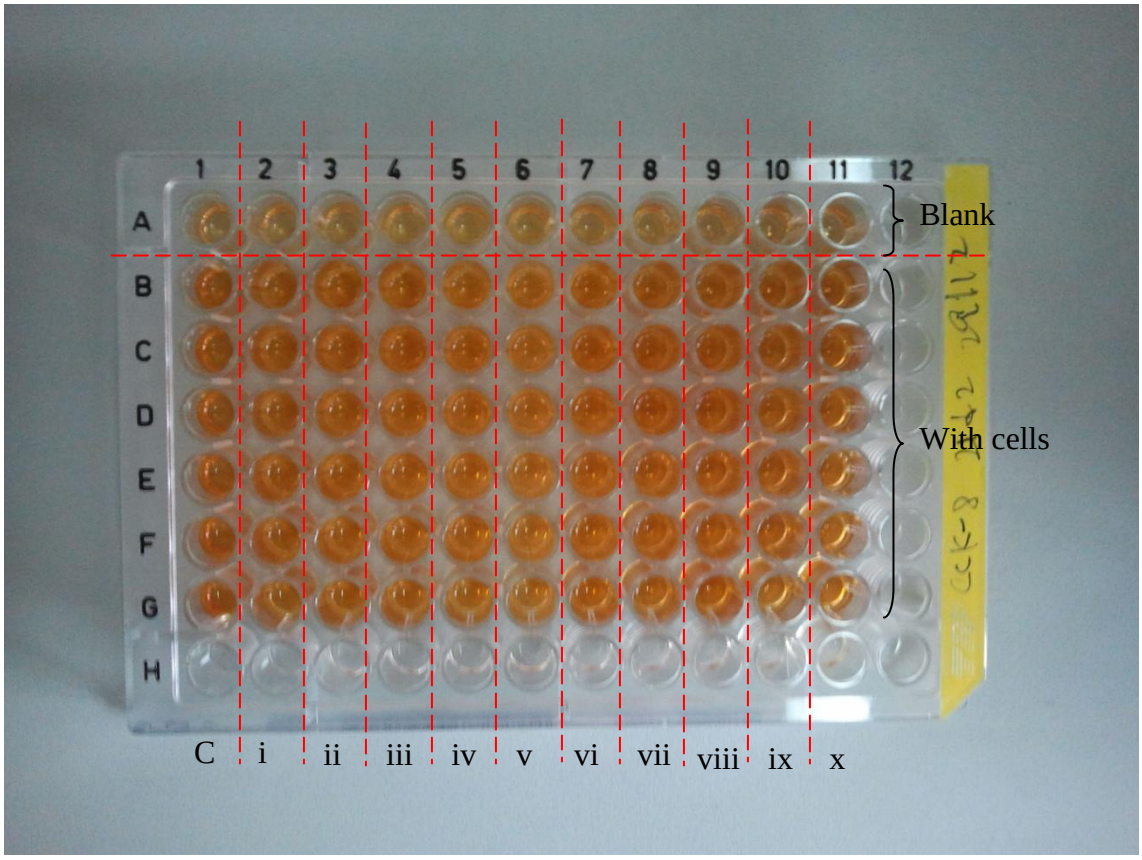


Figure 2.10: Incubation of CCK-8 for 3 hours with live cell produced yellow-orange color. Column A was used as blank wells as the wells were not seeded with cell. i = *D.quercifolia* water extract 10 µg/ml, ii = *D.quercifolia* water extract 50 µg/ml, iii = *D.quercifolia* water extract 150 µg/ml, iv = *D.quercifolia* water extract 250 µg/ml, v = *D.quercifolia* water extract 500 µg/ml, vi = *J.gendarussa* water extract 10 µg/ml, vii = *J.gendarussa* water extract 50 µg/ml, viii = *J.gendarussa* water extract 150 µg/ml, ix = *J.gendarussa* water extract 250 µg/ml, x = *J.gendarussa* water extract 500 µg/ml and C = control. *n* = 6.

2.5.3 ALP detection for rBMS derived osteoblast incubated with various concentration of *D.quercifolia* and *J.gendarussa* water extract

The steps for ALP detection were same as discussed in section 2.4.3. Similar steps were employed for cell lysate sample acquisition as well as total protein content and ALP activity determination. The cell seeding number was 1 x 10^4 cell/well.

2.6 STATISTICAL ANALYSIS

Statistical analysis was performed by using One-Way ANOVA test to compare the means of samples and control values on following tests:

(a) The mean values of proliferation rate of rBMS derived osteoblasts at each interval of incubation with herbal extracts and control,

(b) The mean values of ALP activity of rBMS derived osteoblasts at each interval of incubation with herbal extracts and control,

C

(c) The mean values of osteocalcin production of rBMS derived osteoblasts at each interval of incubation with herbal extracts and control.

All values were expressed as Mean $\pm$ SD. Experiments were performed at least in 6 replicates (n=6) and results of representative experiments were presented except where otherwise indicated.

CHAPTER 3:

RESULTS

3.1 SOLVENT EXTRACTION OF MEDICINAL HERBS

Extraction of medicinal plant was done in Tissue Engineering Laboratory of Department of Biomedical Engineering by solvent extraction method. Four solvent were used in order to extract compounds with different polarity within *D.quercifolia, G,vulgaris* and Bo-Gu-Cao formulation and the outcomes were called ethanolic extract, hexane extract, ethyl acetate extract and water extract respectively. The outcomes of solvent extraction on the herbs and formulation are listed in Figure 3.1.

Table 3.1: Outcomes of solvent extraction on *D.quercifolia, J.gendarussa* and Bo-Gu-Cao formulation.

Herb	Extraction	Compound polarity
D.quercifolia	Ethanol	70 % polar + 30 % non-polar
	Hexane	Non-polar
	Ethyl acetate	Semi polar
	Water	Polar
Justicia gendarussa	Ethanol	70 % polar + 30 % non-polar
	Hexane	Non-polar
	Ethyl acetate	Semi polar
	Water	Polar
Bo-Gu-Cao formulation	Ethanol	70 % polar + 30 % non-polar
	Hexane	Non-polar
	Ethyl acetate	Semi polar
	Water	Polar

The extracts appeared in dark or dark-green mass after solvent extraction processes and were weighed with electronic balance in order to estimate yield percentage from raw plants or herbal formulation before the extracts were stored at – 20 ^{o}C in individual screw-cap bottle (as seen in Figure 3.1). The yield percentage of each extracts from respective dried investigated entities was listed in Table 3.2.

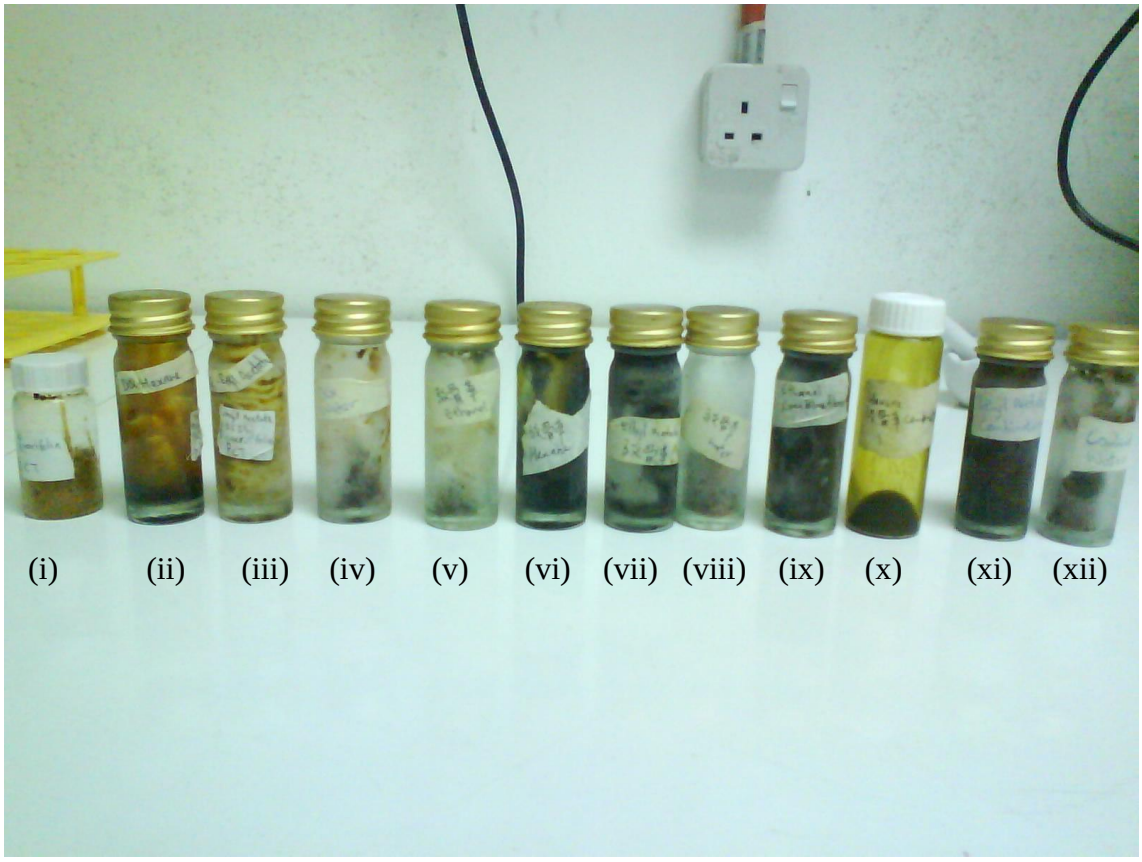


Figure 3.1: Solvent extracts from *D.quercifolia*, *J.gendarussa* and Bo-Gu-Cao formulation were stored in screw-cap bottle. The bottles were kept in – 20 ^{o}C freezer for long term storage. i = *D.quercifolia* ethanolic extract, ii = *D.quercifolia* hexane extract, iii = *D.quercifolia* ethyl acetate extract, iv = *D.quercifolia* water extract, v = *J.gendarussa* ethanolic extract, vi = *J.gendarussa* hexane extract, vii = *J.gendarussa* ethyl acetate extract, viii = *J.gendarussa* water extract, ix = Bo-Gu-Cao formulation ethanolic extract, x = Bo-Gu-Cao formulation hexane extract, xi = Bo-Gu-Cao formulation ethyl acetate extract and xii = Bo-Gu-Cao formulation water extract.

Table 3.2: Yield of herbal extracts from respective dried raw material expressed as weight percentage.

Herbal extract		**Weight (g)**	**Yield (x 100 % of weight)**
D.quercifolia (1.13 kg)	Ethanol	153.86	13.62
	Hexane	43.76	3.87
	Ethyl acetate	61.46	5.43
	Water	47.78	4.23
Justicia gendarussa (1.68 kg)	Ethanol	217.41	12.94
	Hexane	57.91	3.45
	Ethyl acetate	79.18	4.71
	Water	87.16	5.19
Bo-Gu-Cao formulation (1.50 kg)	Ethanol	193.46	12.90
	Hexane	57.12	3.81
	Ethyl acetate	64.78	4.32
	Water	78.14	5.21

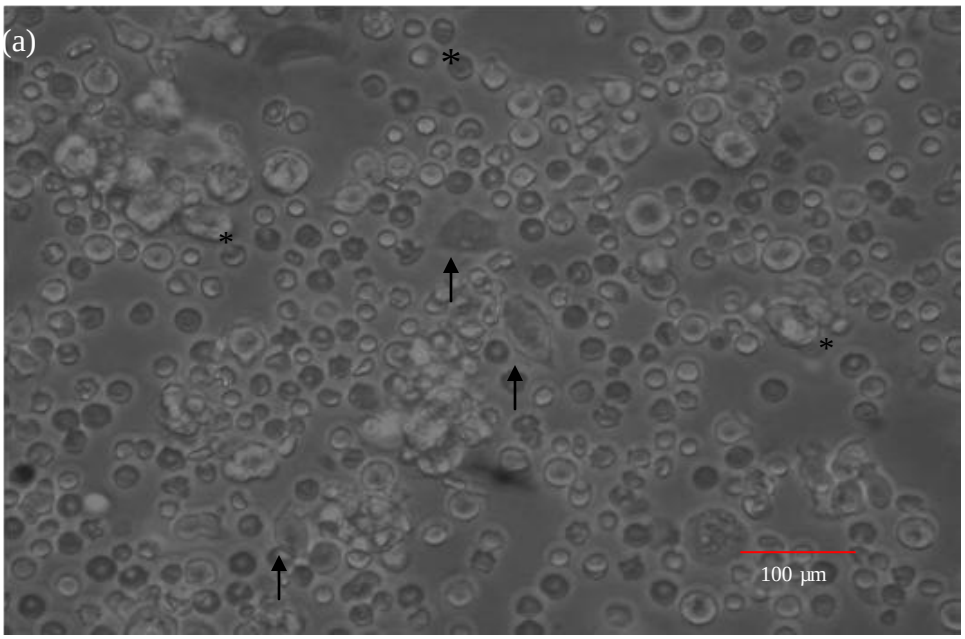
Ethanolic extract was first obtained from dried raw material. Ethanolic extract contains both polar and non-polar compounds. Other extracts were obtained from ethanolic extract according to polarity.

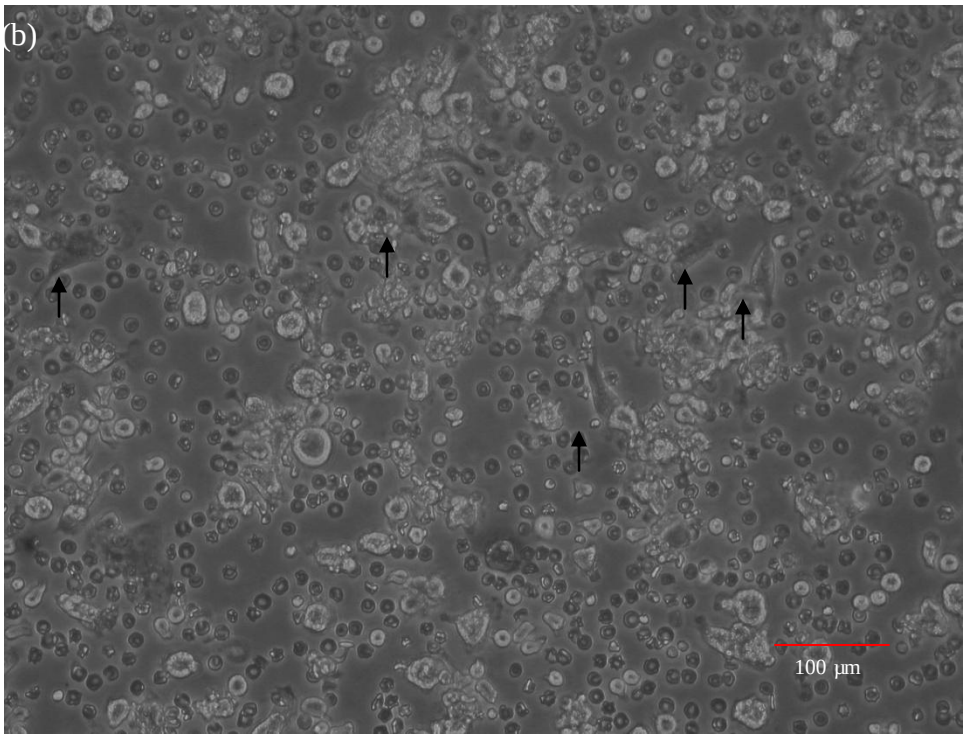
The yield percentage of ethanolic extract for three investigated entities is almost the same, which accounted about 13 % of respective dried raw material. However, partial fractionation by three other solvent accounted differently between *D.quercifolia* and *J.gendarussa*. Ethyl acetate extract of *D.quercifolia* was the highest weight percentage

yield among the three solvent extracts (5.43 %) On the other hand, water extract of *J.gendarussa* accounted highest (5.19 %). The three extracts of Bo-Gu-Cao formulation accounted similar weight percentage yield to *J.gendarussa* whereas the water extract of the formulation yielded highest in term of weight percentage which was 5.21 %.

3.2 rBMSC ISOLATION, CULTURE AND IDENTIFICATION

Adherent cell obtained from fresh isolation of bone marrow cells usually accompanied with hematopoietic cells such as red blood cells, white blood cells and platelets. In this study, the cells were plated in T-75 cm^2 flasks and fed with primary medium after isolation from bone marrow. rBMSC were identified as large spindled cells with nucleus which were different from white blood cells. All the cells were floating in culture flask at this time point (Figure 3.2 (a)). rBMSC need at least hours to adapt themselves in culture flask and attach to plastic surface of the culture flask. The exact attachment time was not studied but the cells in culture flask were left in CO_2 incubator for 4 days. After 4 days of culture, the hematopoietic cells remained unattached to the bottom surface of culture flask as expected. On the other hand attachment of rBMSC to the bottom surface and cytoplasm extension and spreading polygonal morphology were clearly observed (Figure 3.2 (b)). Repeated washes with sterile phosphate buffered solution (PBS) removed non-adherent cells and rBMSC with apparent nuclei and polygonal shape, attached and spread on the bottom surface of culture flask remained (Figure 3.2 (c)). Some rounded and spindled cells with apparent nuclei were observed after repeated wahes. These cells were believed to be the newly formed BMSC. A single cell layer was observed on the bottom surface of culture flask after 2 weeks of culture (Figure 3.2 (d)). These cells had fusiform appearance, with flattened cytoplasm and nuclei. On top of single cell layer, rounded or spindled newly formed BMSC with apparent nuclei were observed.


(a)
100 µm


(b)
100 µm

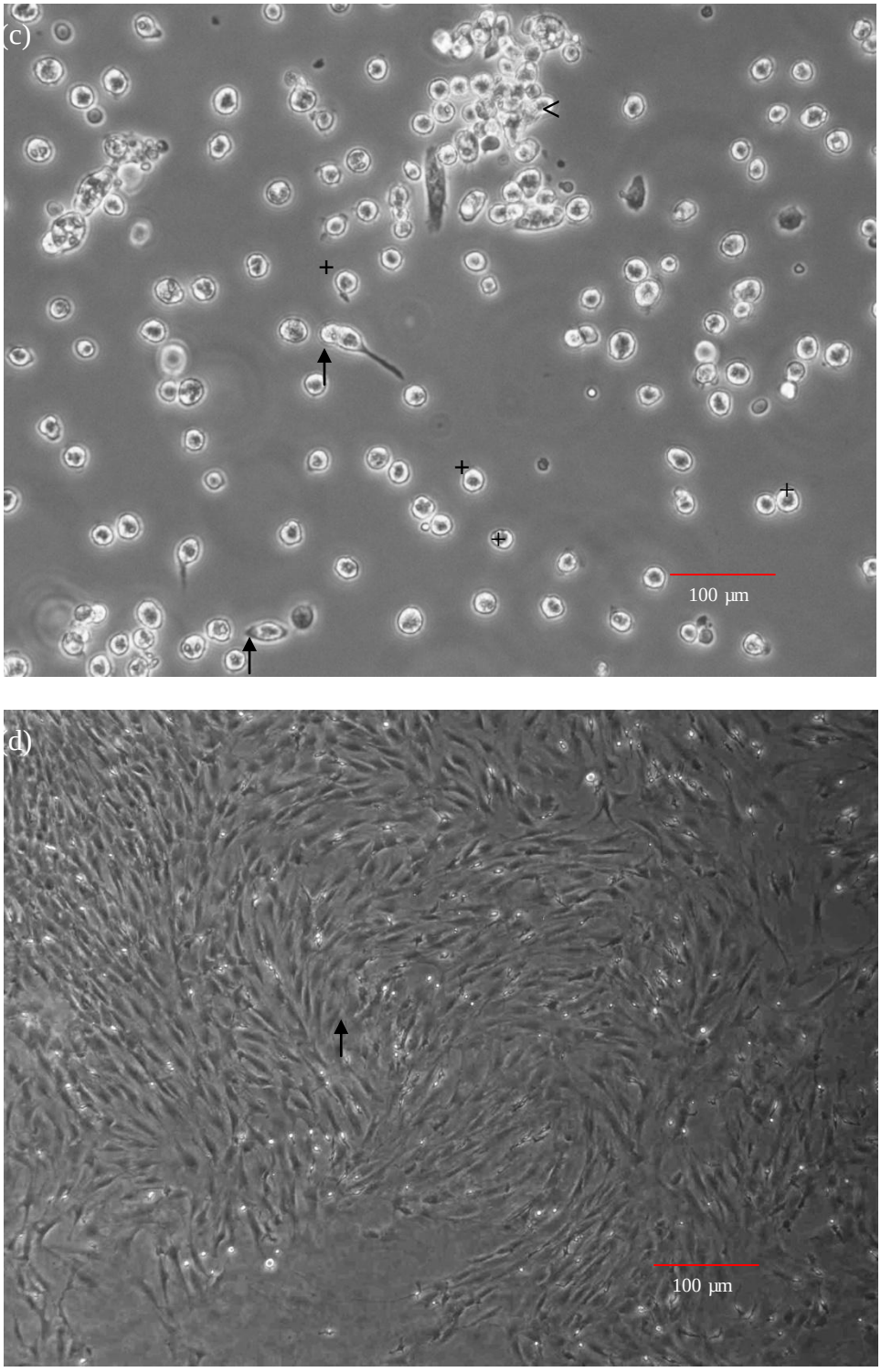


Figure 3.2: Photographs of rBMSC from isolation to cell confluency. (a) Cells from rat bone marrow immediately after isolation. The cells were mix of adherent cells and non-adherent cells. Number of red blood cells was prominent compared to large-spindled rBMSC. (b) The cells were cultured for 4 days after isolation. rBMSC attached to bottom surface and their cytoplasm spread. (c) Adherent cells remained after repeated washes with PBS. Rounded, loosely attached newly formed cells were observed. Aggregate of cells was seen as well. (d) Cell confluency was seen after 2 weeks culture. A single layer cell was

observed. (↑) Attached rBMSC, (*) Red blood cell, (+) Newly formed cells, (<) cells aggregate; inverted microscope; Primary medium; T-75 cm^2 flask.

3.2.1 Characterization of rBMSC

Characterization of rBMSC was done prior to inducing the cells to osteoblasts and to be used as a subject in the investigation of herbal extract effect on the cells. RBMSC are able to be precursor cells for various types of cells. However, the rBMSC have own characteristics which distinct the cells from other animal cell type. The characteristics were to be found in this study to confirm the cells used in this study were not other cells like fibroblasts, myoblasts, adipocytes and others which might present together with stromal cells when isolated from bone marrow.

a) Fibroblastic appearance and adherent to plastic surface:

   Both features were observed under an inverted microscope. The cells appeared in spindled shape, either in bipolar or multipolar. Generally, the cells were elongated after adhered to plastic surface of culture flask. Cell adherent feature was confirmed by immobility of fibroblatic-like cells after gentle shaking of culture flask.

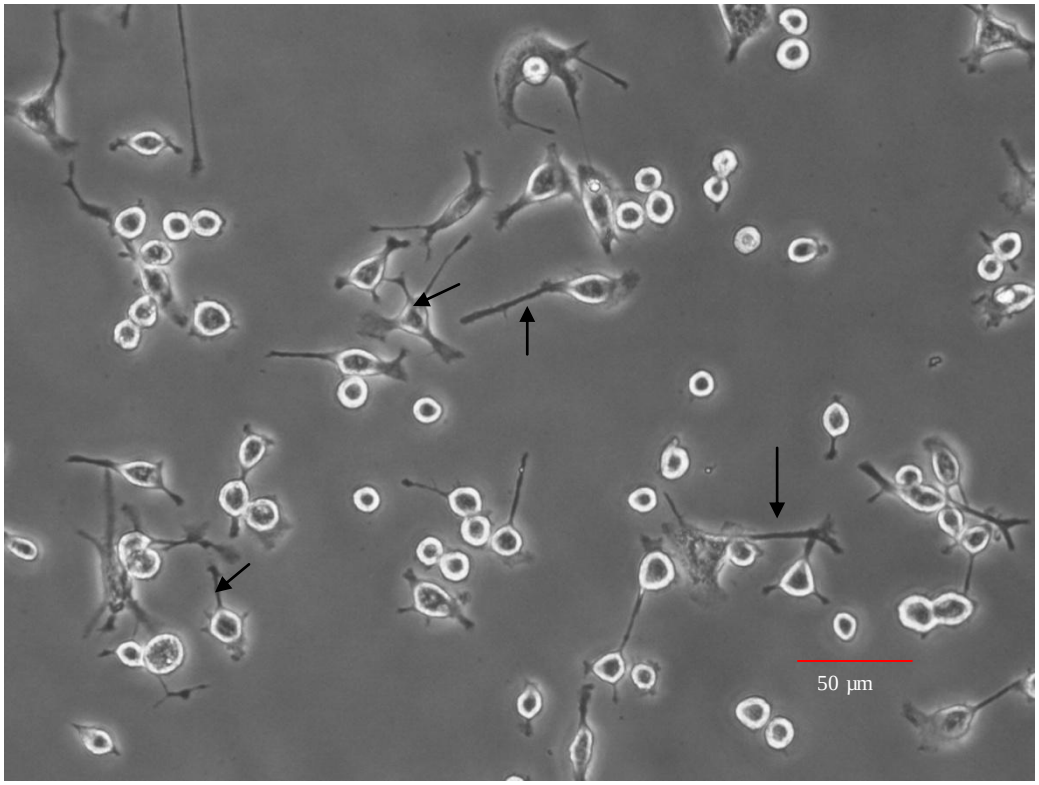


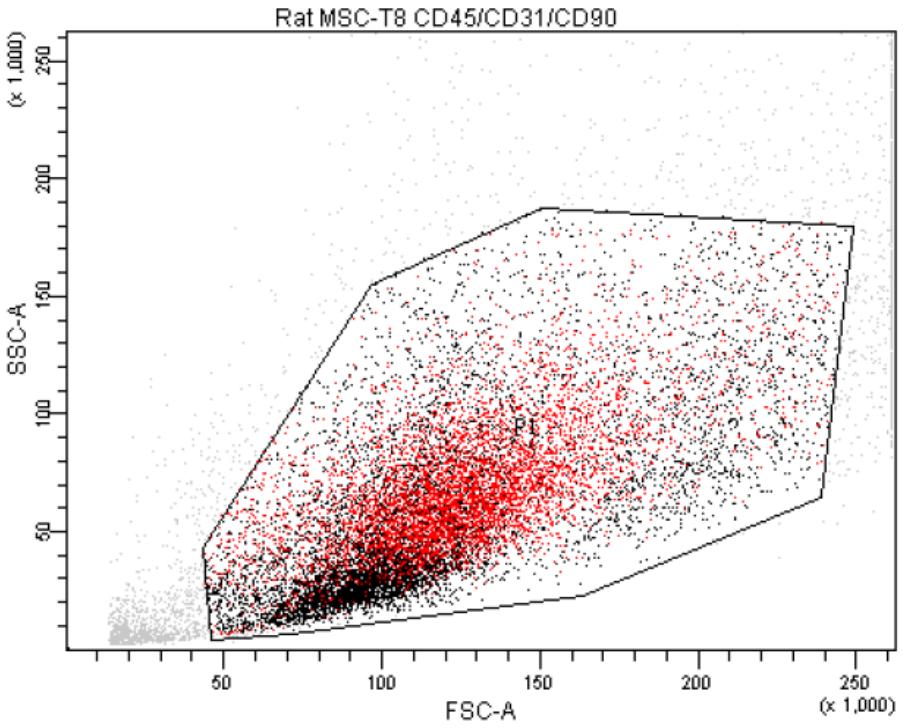
Figure 3.3: The photograph of rBMSC after 6 days in primary medium. Elongated/spindled cytoplasms (bipolar/multipolar) were observed (↑).

b) Mesenchymal stem cells markers confirmation:

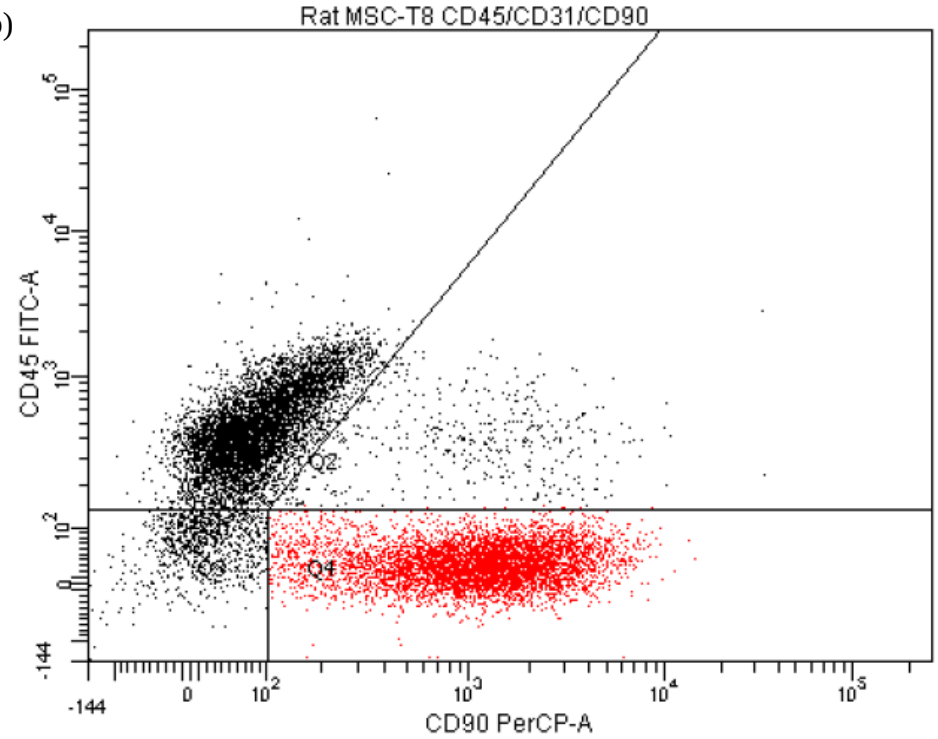
CD90+, CD31- and CD45- were identified using flow cytometry to confirm mesenchymal stem cells. CD90 is the marker can be found on mesenchymal stem cell while CD31 and CD 45 are those to be found in hematopoietic cells. Presentation of CD90+, CD31- and CD45- is enough to confirm mesenchymal stem cell and rules out hematopeietic cell (which could culture along after rBMSC isolation) .SSC (Side scatter) and FSC (Forward scatter) light dispersion properties allow a good discrimination between viable (fall within polygon) and dead cells or cell debris (fall out of polygon). There were two populations of cells detected in the SSC-A versus FSC-A plot, with one represented by black color, and another one represented by red color (Figure 3.4 (a)). The cells were further analyzed for CD90 plot (Figure 3.4 (b)). Cell population represented by red color was shown positive for CD90 marker. Another cell population which was represented by black color was detected

as CD90-. Markers CD31 and CD 45 were checked on CD90+ population. The outcome is shown in Figure 3.4 (c). Both CD31 and CD 45 were absent in CD90+ population. Thus, the cell population represented by red color was mesenchymal stem cell obtained from rat bone marrow and presented in the cell cultures in this study.

(a)



(b)



(c)

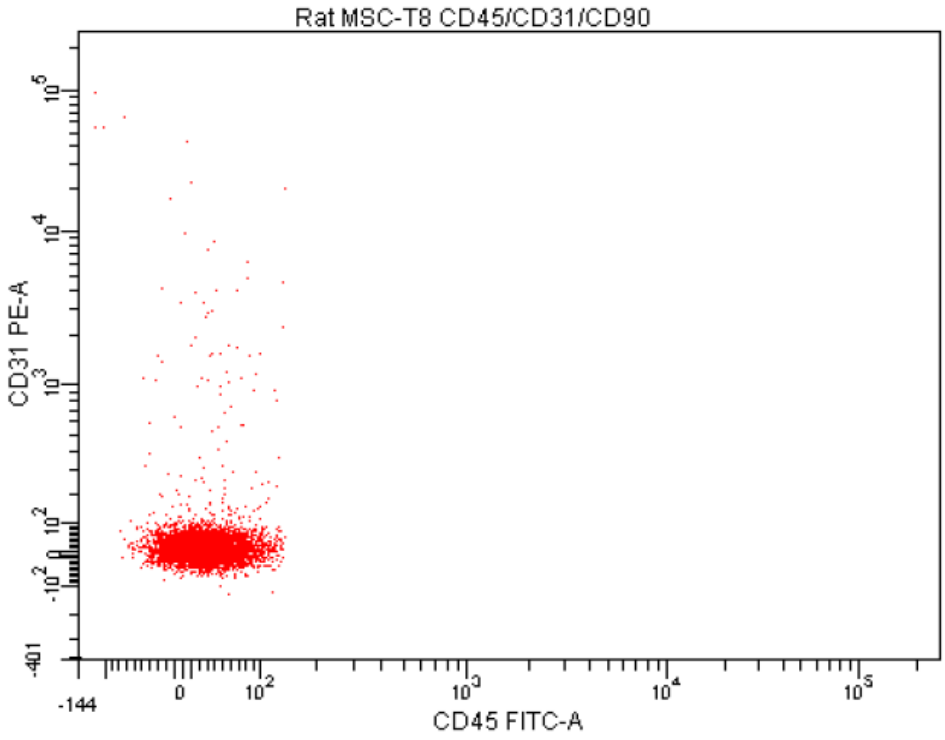


Figure 3.4: Flow cytometry results of rBMSC at passage 1. (a) SSC-A (cell granulation) versus FSC-A (cell size) indicates cell distribution. (b) Cells represented by red color in quadrant 4 (Q4) confirms present of CD90+ cells. (c) Red color representing cells were confirmed CD31- and CD45-.

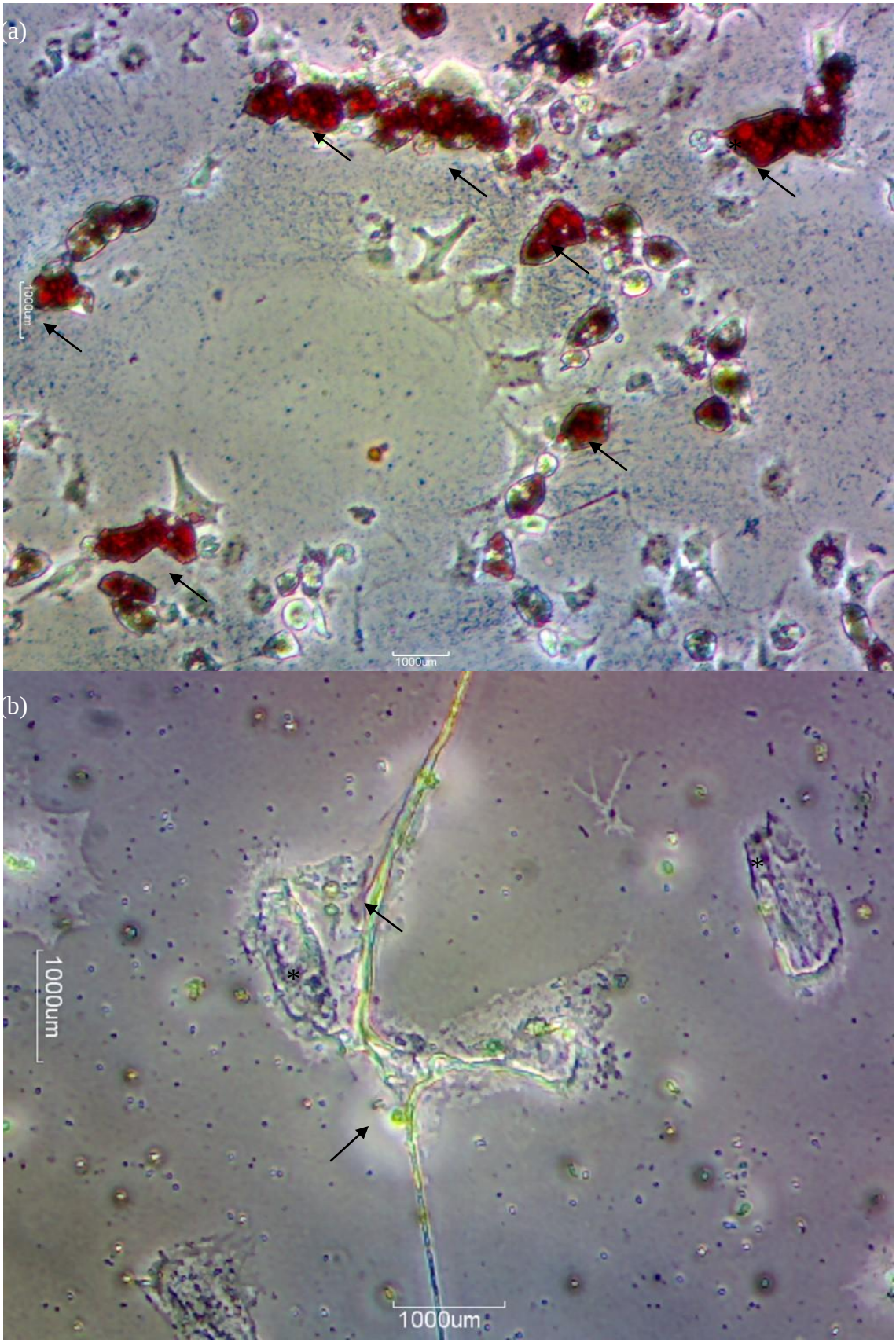
c) Multilineages differentiation:

The rBMSCs were induced to 3 lineages differentiation under proper induction growth factor respectively for 21 days.

i. **Adipogenesis**: As shown in figure 3.5 (a), the cells were stained with Oil Red O staining method. The oil droplets within cells were staied red and confirmed the adipogenesis.

ii. **Chondrogenesis**: The cells were induced to differentiate into chondrocyte as indicated in figure 3.5 (b), although the staining was not really excellent. The cartilage was stained by Safranin O as yellow-orange color and considered stained very mild as the cartilage should be stained in orange-red color. The cytoplasm was stained bluish green by fast green dye. However, chondrogenesis was proven by production of proteoglycans.

iii. **Osteogenesis**: Alizarin Red S staining was used in this study to detect osteoblasts (osteogenesis of rBMSCs). As shown in figure 3.5 (c), the red color was prominent in the staining result and indicated deposition of calcium, an important indicative sign of presence of osteoblasts.


(a)
1000um
1000um
(b)
1000um
1000um

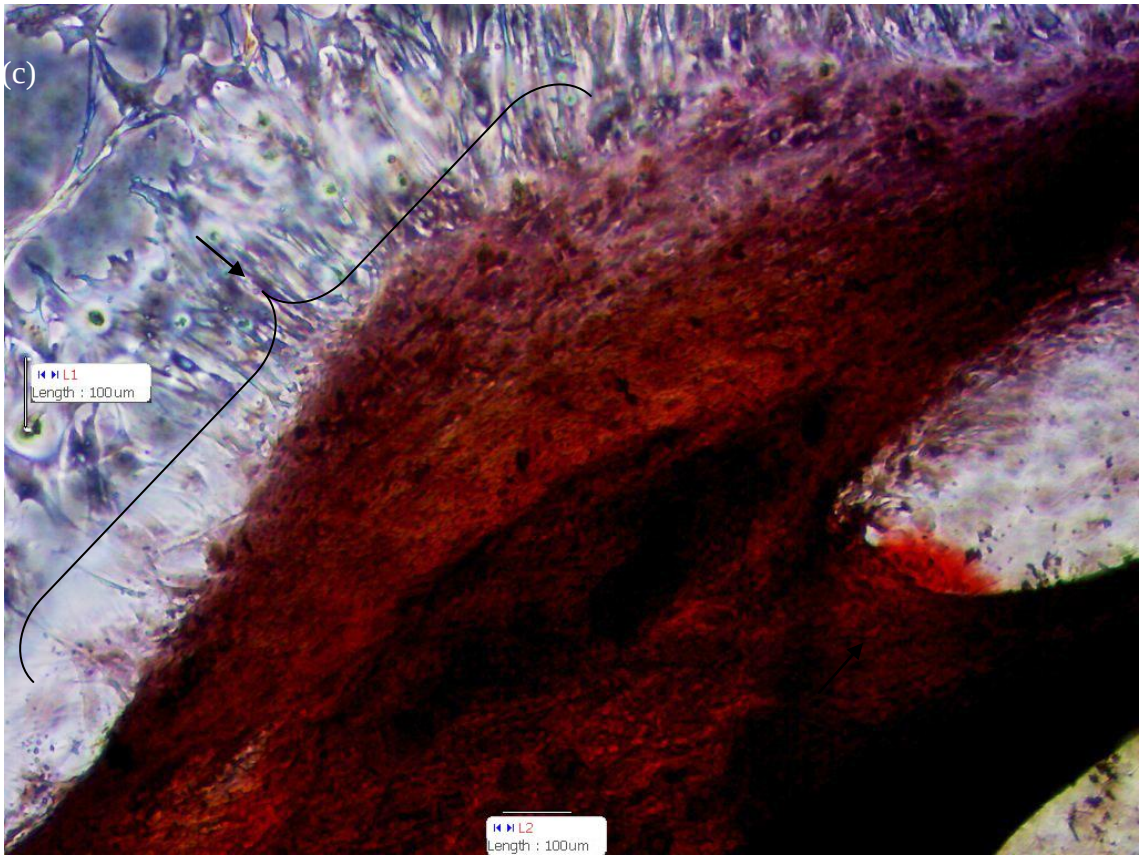
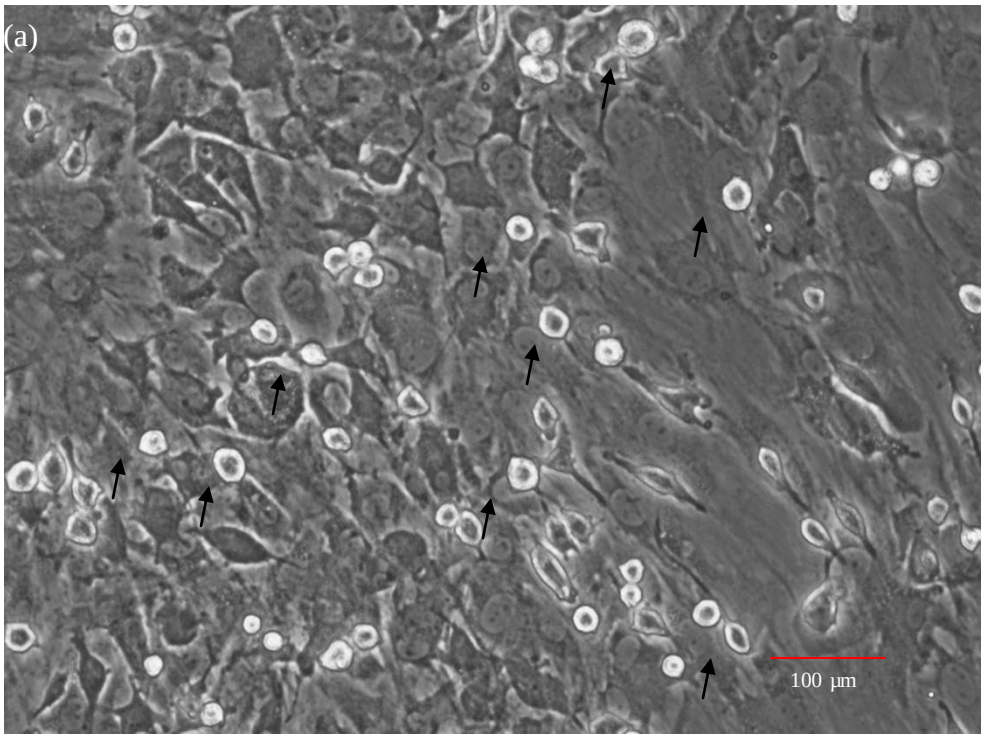


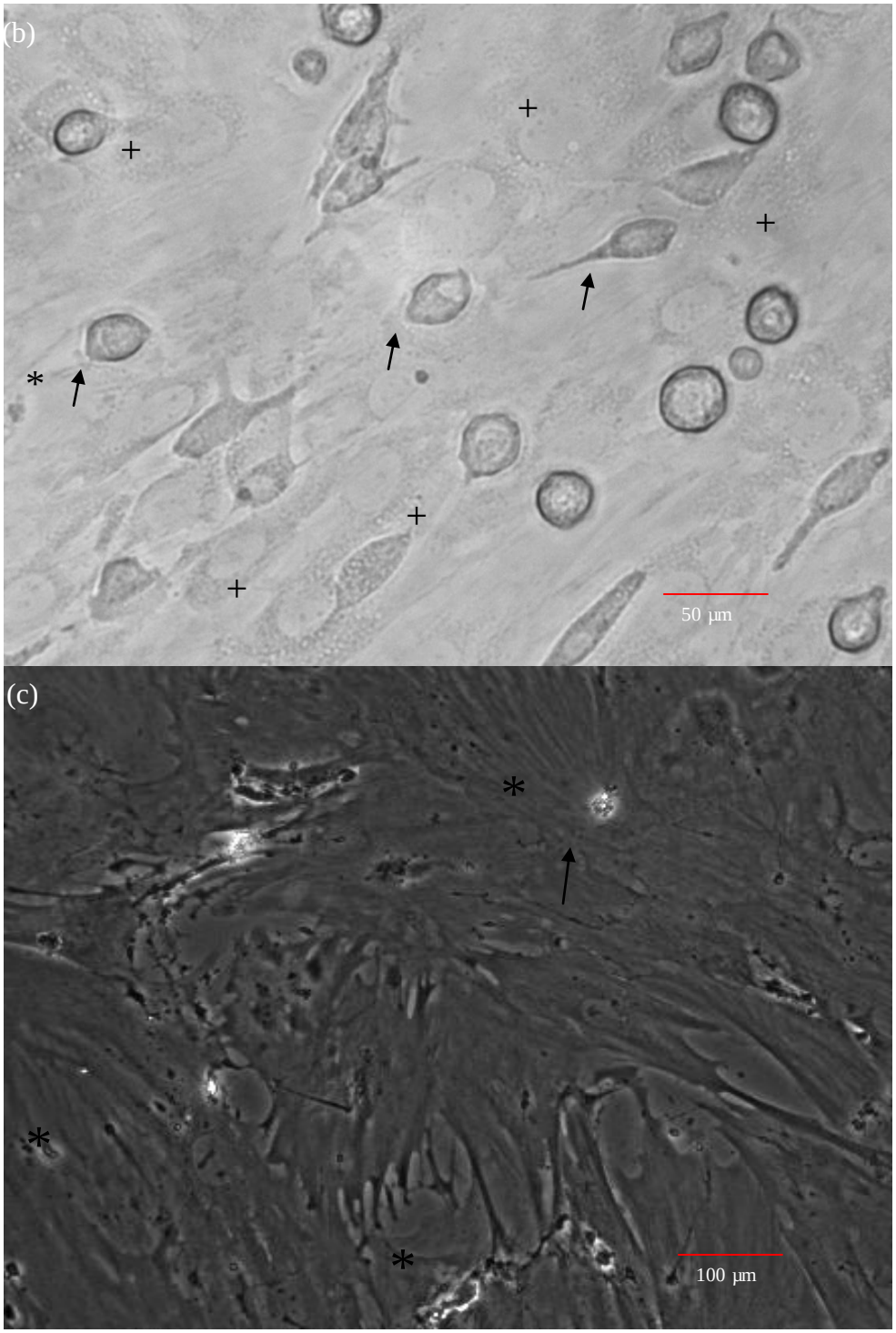
Figure 3.5: Three lineages differentiation was evaluated qualitatively by respective staining methods. (a) Oil Red O staining stained fat vacuole with red color (↑)Fat vacuole. (b) Safranin O staining stained cartilage production with yellow color. Cytoplasm was stained bluish green (↑)Cartilage, (*)Cytoplasm. (c) Calcium deposits was stained red by Alizarin Red Solution (↑)Calcium deposits.

3.2.2 rBMS derived osteoblasts culture

To induce osteogenic differentiation of rBMSC which to be used as subject in this study, osteogenic medium (DMEM/F12 with 10 % FCS, 1 % Atibiotic-antimycotic solution, 10 nM Dexamethasone, 5 mM β-GP, 50 μg/ml L-ascorbic acid) was fed to rBMSC instead of primary medium 4 days after isolation. BMSC grew into single cell layer in osteogenic medium. At this stage, the cells were irregular or cuboidal in shape and highly packed, with flatted cytoplasm and rounded nuclei (Figure 3.6 (a) and (b)). The cells at 2nd passage onwards were characterized with the formation of massive extracellluar

matrix and mineral nodules deposition. After one month, the mature osteoblast taken from rBMSC culture showed multi-possesses morphology secreting extracellular matrix with mineral nodule deposition (Figure 3.6 (c)). However, observation of the cells under inverted microscope directly was not enough to confirm the osteogenesis of rBMSC. Alizarin Red S staining was done on the culture and result was shown in figure 3.6 (e). The cells were stained in red color and proven the calcium deposition by osteoblasts (rBMS derived osteoblast). In cell harvesting for assays, accutase was used to digest all proteins or the extracellular matrix from single cell layer to free the cells (Figure 3.6 (d)). Each cell became rounded in shape and slowly detached from the bottom surface of culture flask.




(b)
50 µm
(c)
100 µm

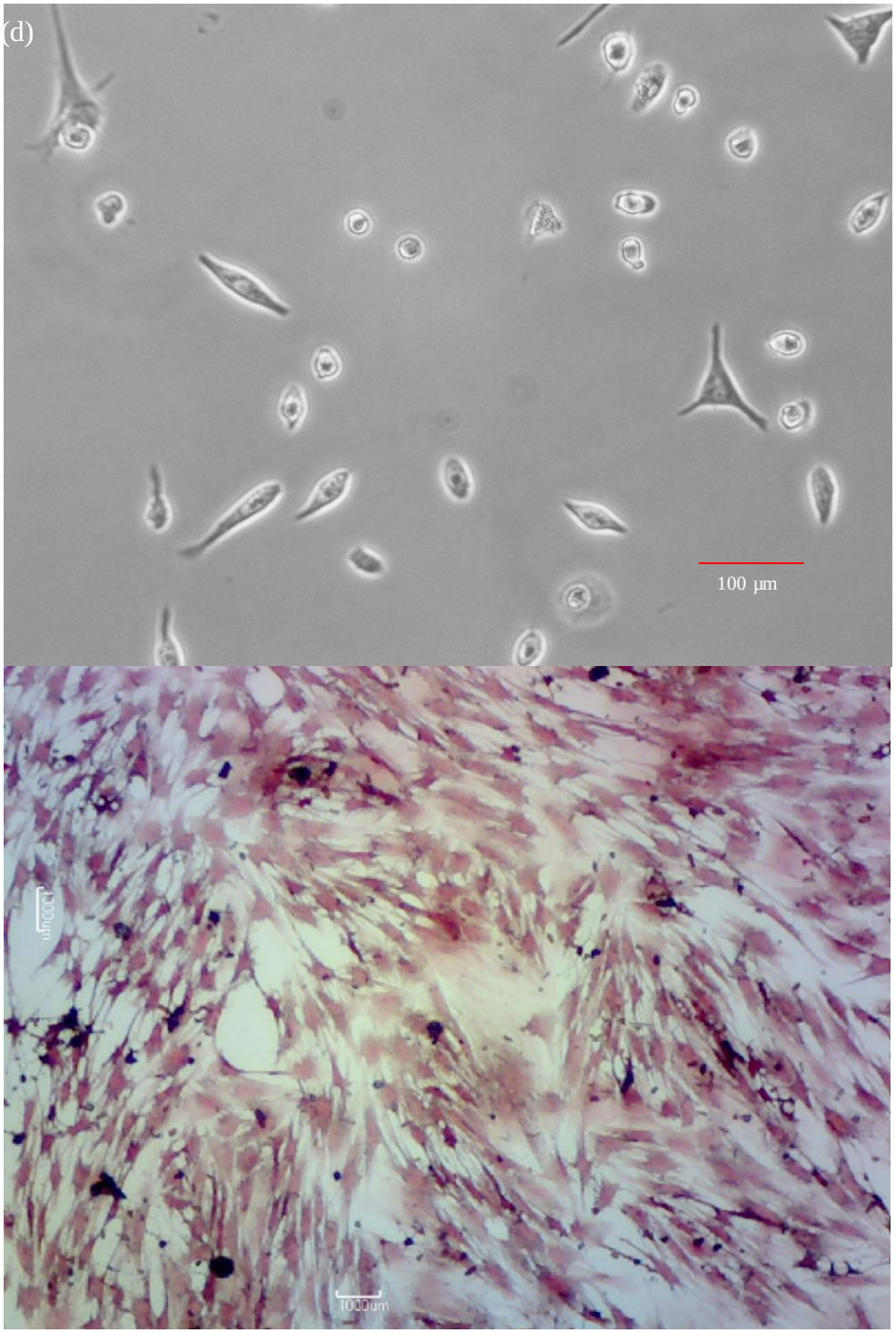


Figure 3.6: RBMSC cultured in osteogenic medium. (a) RBMSC appeared in cuboidal or irregular shape. (b) Flattened cytoplasm was seen in confluent cell culture under high magnification microscope. (c) Multi-processes morphology secreting extracellular matrix was observed. Black dots were calcium deposits. (d) Round cells were observed after incubated with accutase. Extracellular matrix was digested. (e) Alizarin Red Solution stained calcium deposits red to confirm osteogenesis. (↑) Nucleus, (+) cytoplasm.

3.3 SCREENING OF EFFECT OF BIOCHEMICAL ENHANCER ON rBMS DERIVED OSTEOBLAST

3.3.1 Generating standard curve of cell number

A standard curve was generated using OD of formazan produced by dehydrogenizing WST – 8 in CCK – 8 Kit solution against known cell number. A strong linear correlation was seen in the range of $2 \times 10^3 – 2.4 \times 10^4$ cell/well between absorbance and cell number. Equation obtained from the standard curve is $Y = 8 \times 10^{-5} X$ where OD = Y, cell number = X, $R^2 = 0.9553$. The standard was used to estimate cell number after incubated with herbal extract. The estimation of cell number is based on the dehydrogenase activity detection in viable cells, e.g. cell number = intensity of color of formazan. Note that conditions or chemicals that affect dehydrogenase activity in viable cells may cause discrepancy between the actual viable cell number and the cell number determined using the CCK-8 assay.

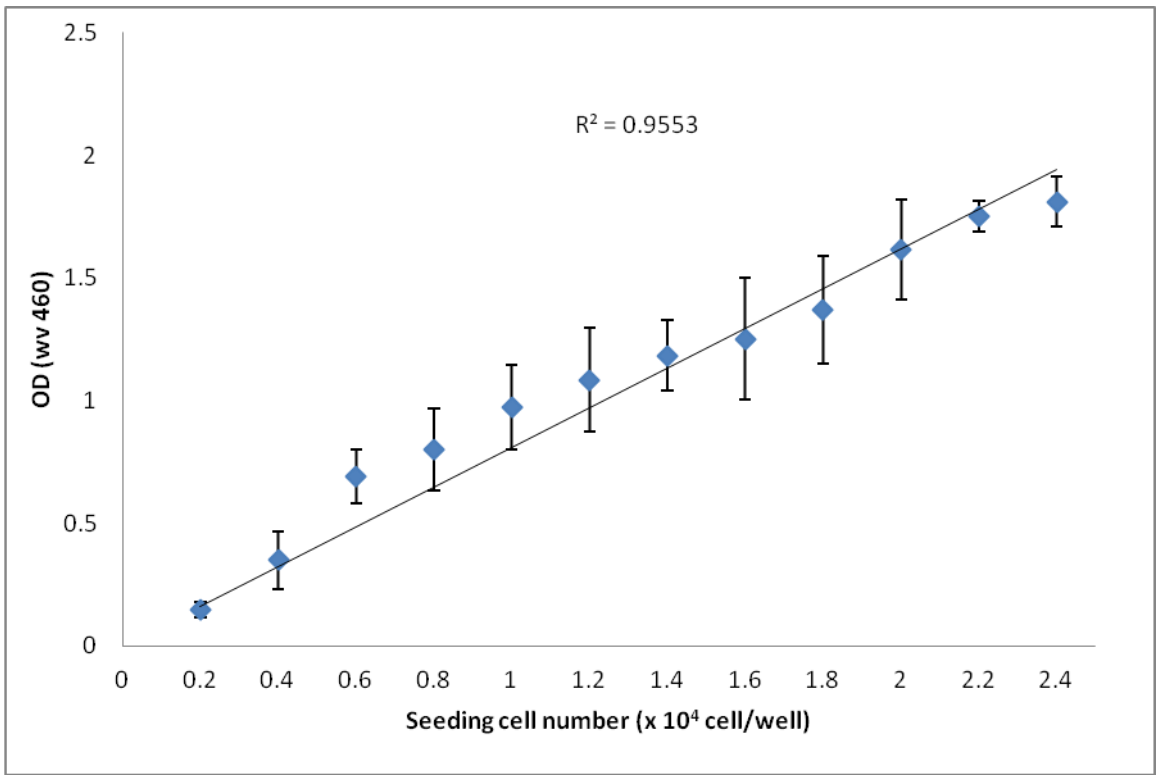


Figure 3.7: The relationship between the seeding number of cells per well and the OD values of the formazan produced. After incubation for 6 hours, the formazan produced by viable cells were quantitated using the CCK – 8 Assay (incubation period = 3 hours). OD values measured were proportional to the seeding number of rBMS derived osteoblasts seeded per well ranged from 2 x 10^3 – 2.4 x 10^4 cell/well. Each data point represented mean OD values from 6 duplicates wells ± SD.

3.3.2 Proliferation rate of rBMS derived osteoblasts after incubation with herbal extract

The proliferation rate of rBMS derived osteoblasts was evaluated by comparing the cell number after 3, 7 and 14 days incubation with herbal extract to control cell (cell incubated with osteogenic medium only).

1. ***D.quercifolia***

Proliferation evaluation of rBMS derived osteoblasts incubated with *D.quercifolia* showed no significant difference between every single type of herbal extract and control after 3 days incubation. Cells incubated with ethanolic extract proliferated 22.0±7.98 %

lower than control. Water extract of *D.quercifolia* induced proliferation at 9.5±18.11 % higher than control, followed by ethyl acetate extract (7.0±11.23 %) and hexane extract (5.3±11.12 %).

After the cells being incubated with *D.quercifolia* extracts for 7 days, the cells incubated with hexane extract proliferated 55.6±6.32 % higher than control, which is the highest among the 4 type of extracts against control. Water extract induced significant difference in proliferation compared to control as well, at 26.2±8.73 %. Ethyl acetate extract induced non-significant enhancement, which is at 19.2±8.77 %. Ethanolic extract proliferated 16.9±3.97 % less than control. However the difference is not significant.

Hexane extract induced highest enhancement among 4 types of *D.quercifolia* extracts again after 14 days. Ethyl acetate and water extract recorded mild but not significant enhancement (7.7±6.32 % and 29.3±10.18 % respectively). Ethanolic extract was again proliferating less than control. After 14 days incubation, the cells showed 22.0±6.62 % less than control cells (Figure 3.8 (a)).

2. *Justicia gendarussa*

All 4 types of extract of *J.gendarussa* did not enhance rBMS derived osteoblasts significantly compared to control after incubation with respective herbal extract. Ethanol, ethyl acetate and hexane extracts showed similar enhancement on rBMS derived osteoblasts, which were 32.2±29.29 %, 32.7±26.41 % and 33.4±29.07 % respectively. Water extract enhanced proliferation of rBMS derived osteoblasts the least among 4 types of extract (8.0±20.71 %).

After incubation with *J.gendarussa* extracts for 7 days, rBMS derived osteoblasts incubated with ethyl acetate extract showed highest enhancement at 93.7±14.31 %, followed by hexane extract (83.0±9.24 %), ethanolic extract (78.3±6.12 %) and water

extract (73.8±9.62 %). Four types of extracts induced proliferation enhancement compared to control significantly.

Water extract recorded highest enhancement percentage among 4 types of extract in proliferation compared to control after 14 days incubation. The rBMS derived osteoblasts were enhanced significantly at 47.7±25.80 %. Ethyl acetate and water extract enhanced the proliferation of the cells significantly as well at 37.2±18.12 % and 30.9±12.95 % respectively. Hexane extract enhanced the cells proliferation by 27.1±13.08 % but the enhancement was not significant (Figure 3.8 (b)).

3. Bo-Gu-Cao formulation

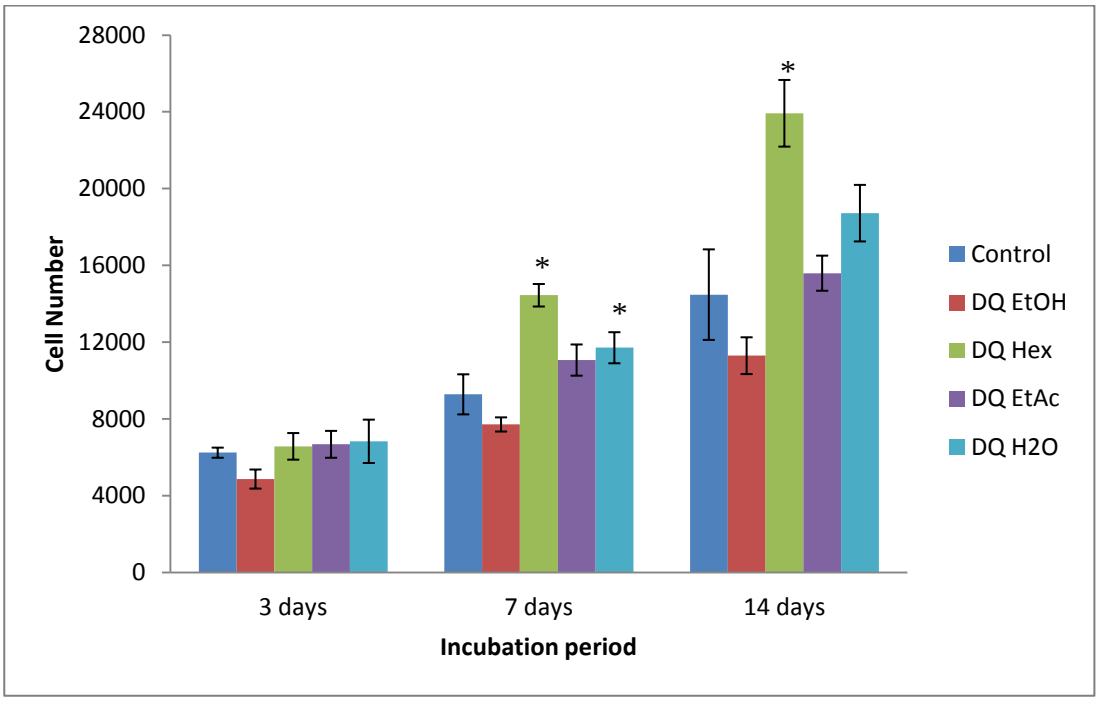
Bo-Gu-Cao formulation extracts did not enhance rBMS derived osteoblasts significantly. Hexane extract showed highest enhancement compared to control by 18.4±5.64 %, closely followed by ethyl acetate extract (17.7±6.98 %) and water extract (17.5±4.64 %). Ethanolic extract showed lowest enhancement but the difference to another 3 extracts was not remarkable. The enhancement percentage was 14.3±10.37 %.

All 4 types of Bo-Gu-Cao formulation extracts enhanced rBMS derived osteoblasts significantly after 7 days incubation. Hexane extract recorded 79.5±11.72 % enhancement compared to control cells, which was the highest among 4 extracts. Ethyl acetate, water and ethanolic extracts enhanced proliferation of rBMS derived osteoblasts by 63.6±16.13 %, 63.4±21.31 % and 61.1±8.04 %.

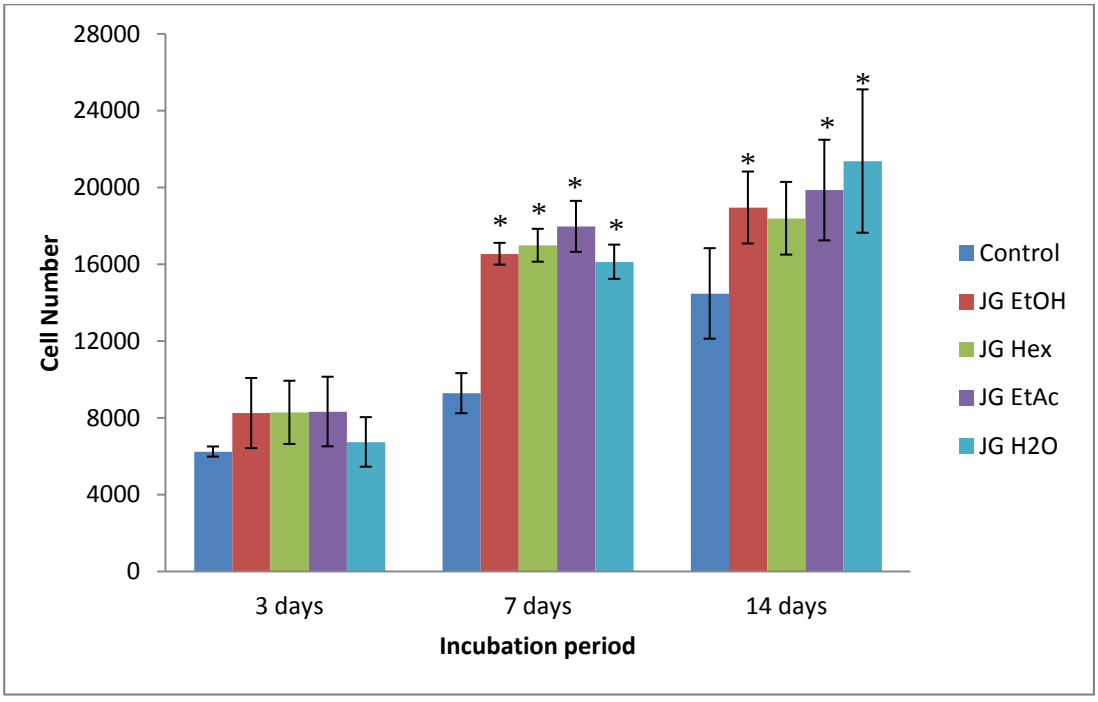
Generally, proliferation enhancement percentage compared to control by Bo-Gu-Cao formulation extracts at 14 days was lower than 7 days incubation. Water extract and ethanolic extract induced significant enhancement compared to control. The enhancement was 42.7±11.36 % and 31.4±10.13 % respectively. Hexane extract and ethyl acetate extract

enhanced the proliferation by 30.8±23.57 % and 23.4±20.60 % respectively. Both enhancements were not significant (Figure 3.8 (c)).

(a)



(b)



(c)

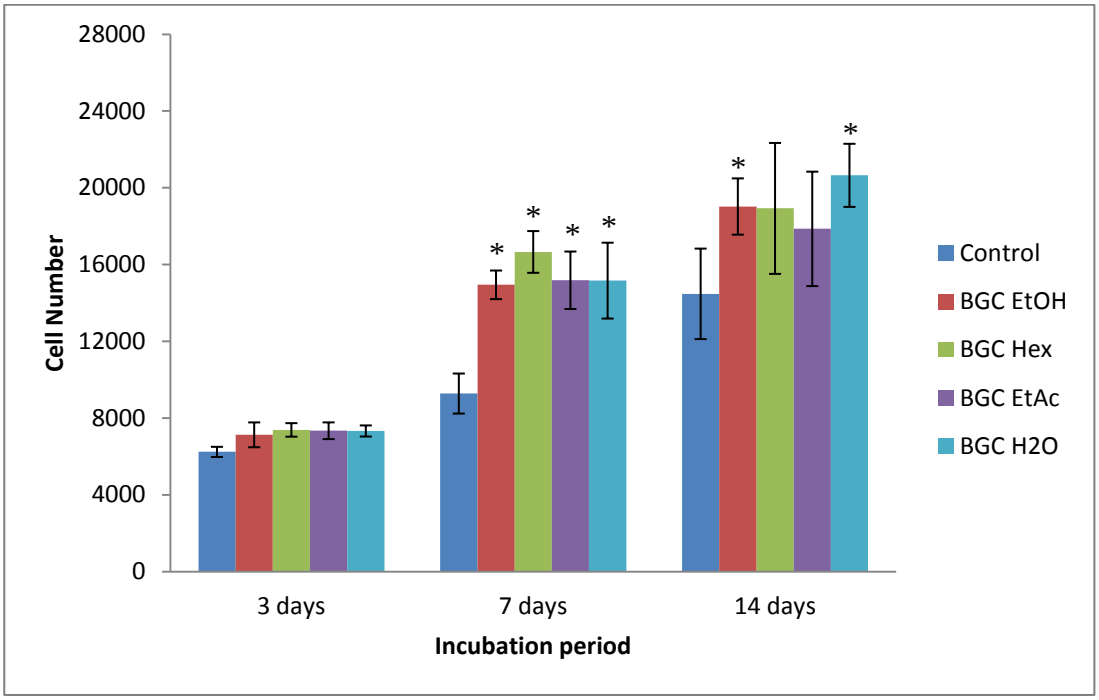


Figure 3.8: Proliferation of rBMS derived osteoblasts after incubated with herbal extracts for 3, 7 and 14 days is shown. Control represents rBMS derived osteoblasts incubated with control solution in osteogenic medium. Values shown are Mean cell number ± SD (n=6); *: $p<0.05$ enhancement over control. DQ EtOH = *D.quercifolia* ethanolic extract, DQ Hex = *D.quercifolia* hexane extract, DQ EtAc = *D.quercifolia* ethyl acetate extract, DQ H_2O = *D.quercifolia* water extract, JG EtOH = *J.gendarussa* ethanolic extract, JG Hex = *J.gendarussa* hexane extract, JG EtAc = *J.gendarussa* ethyl acetate extract, JG H_2O = *J.gendarussa* water extract, BGC EtOH = Bo-Gu-Cao formulation ethanolic extract, BGC Hex = Bo-Gu-Cao formulation hexane extract, BGC EtAc = Bo-Gu-Cao formulation ethyl acetate extract and BGC H_2O = Bo-Gu-Cao formulation water extract. Control value for 3 days incubation = 6238 ± 264 cell/well, control value for 7 days incubation = 9280 ± 1044 cell/well, control value for 14 days incubation = 14476 ± 2359 cell/well.

3.3.3 Generating standard curve of 4-nitrophenol

A standard curve of OD plotted against 4-nitrophenol concentration was generated and used in estimation of ALP activity of sample. In ALP detection assay, pNPP substrate was incubated with cell lysate. ALP in cell lysate converted pNPP substrate to p-nitrophenol. By comparing the OD obtained from reaction mixture to standard curve of 4-nitrophenol, quantity of pNPP substrate converted to p-nitrophenol over time was estimated. Equation obtained from the standard curve is $Y = 23.123 X + 0.0425$ where OD = Y, 4-nitrophenol concentration = X, $R^2 = 0.9904$.

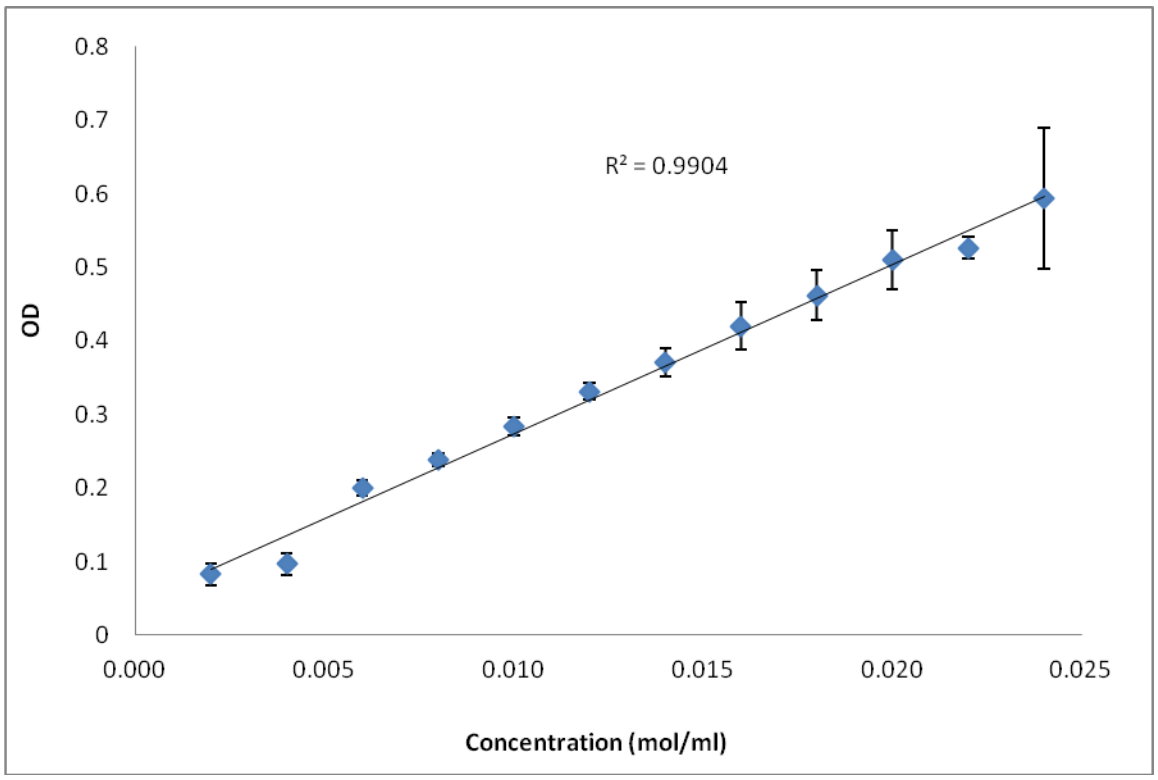


Figure 3.9: The relationship between the concentration of 4-nitrophenol and the OD values. The OD values was read immediately after equivalent amount of 4-nitrophenol dissolved completely in distilled water. OD values measured were proportional to the color intensity produced by respective concentration of 4-nitrophenol ranging from 0.02 – 0.24 mol/ml. Each data point represented mean OD values from 7 duplicates wells ± SD.

3.3.4 ALP Activity determination

ALP is an important indicator for osteoblastic behavior of cell. Effect of herbal extract on ALP activity of rBMS derived osteoblasts was assessed by comparing the ALP activity after 7, 14 and 21 days incubation with herbal extract to respective control cell (cell incubated with osteogenic medium and equivalent amount of DMSO only). ALP activity was determined by normalizing amount of pNPP substrate converted by ALP in certain period to total protein content of cell.

1. *D.quercifolia*

rBMS derived osteoblasts incubated with four *D.quercifolia* extracts showed different response in term of ALP activity after 7 days incubation. Water extract of *D.quercifolia* enhanced ALP activity of rBMS derived osteoblasts as much as 54.4±33.97 % compared to control. The enhancement is significant. However, there is no significant difference between control and the other 3 types of herbal extract after 3 days incubation. ALP activity of cells incubated with ethanolic extract was shown 28.8±24.94 % higher than control. The hexane extract induced non-significant enhancement on ALP activity as well (16.5±16.87 %). ALP activity of cells incubated with ethyl acetate extract was 2.3±22.22 lower than control. However the downregulation of ALP activity was not significant.

After 14 days incubation, the cells incubated with all 4 types of extracts were shown downregulation on ALP activity compared to control. In aligned to the result in 7 days incubation, ethyl acetate extract downregulated ALP activity the most amongst *D.quercifolia* extracts, as much as 51.9±15.77 %. The downregulationof ALP activity of rBMS derived osteoblasts incubated with ethanol, hexane and water extracts was almost the same. Water extract downregulated 40.0±9.03 %, followed by hexane extract (39.8±14.87 %) and ethanolic extract (36.6±20.70 %). The downregulation on ALP activity of rBMS derived osteoblasts by 4 types of *D.quercifolia* extracts was significant.

Interestingly, rBMS derived osteoblasts exhibited ALP acitivity enhancement after 21 days incubation with *D.quercifolia* extracts. This is reversal to the result exhibited in 14 days incubation. Ethanol and water extracts enhanced ALP activity of rBMS derived osteoblasts significantly, which showed 46.2±4.62 % and 51.5±10.95 % enhancement compared to control respectively. Hexane and ethyl acetate extracts didn't show significant enhancement of ALP activity after 21 days incubation. The ALP activity of cells incubated

with hexane extract was 26.4±24.21 % higher than control, whereas ethyl acetate extract was 32.2±7.35 % higher than control. (Figure 3.10 (a)).

2. *Justicia gendarussa*

Water extract of *J.gendarussa* remarkably enhanced ALP activity of rBMS osteoblasts compared to control after 7 days incubation. The enhancement was significant which exhibited ALP activity of 111.4±36.60 % higher than control. Contrary to enhancement shown in cells incubated with water extract, hexane extract showed significant downregulationon ALP activity of rBMS derived osteoblasts after 7 days incubation. The ALP activity was 53.0±36.64 % lower than control. Ethanol and ethyl acetate extracts have shown mild and non-significant downregulationon ALP activity of rBMS derived osteoblasts, which were 2.1±16.60 % and 1.5±22.29 % lower than control respectively.

The enhancement/downregulationof ALP activity observed in rBMS derived osteoblasts incubated with *J.gendarussa* extracts for 14 days compared to control was agreed to result shown in 7 days incubation. Water extract was the only one in 4 types of extracts which enhanced ALP activity of rBMS derived osteoblasts after 14 days incubation. The enhancement was 16.6±16.38 % compared to control. However, the enhancement was not significant. The other 3 extracts induced significant downregulationon ALP activity in rBMS derived osteoblasts after 14 days incubation. Hexane extract downregulated the most with 66.0±4.76 % downregulation, followed by ethyl acetate (55.2±7.42 %) and ethanol (34.3±8.52 %) extracts.

In 21 days incubation assay, water extract was again the only herbal extract that enhanced ALP activity of rBMS derived osteoblasts. The enhancement was 43.5±22.72 % and significant. Hexane extract was the only herbal extract that downregulated ALP

activity of rBMS derived osteoblasts significantly.The downregulationwas 41.1±11.38 % compared to control. The ALP activity of the cells incubated with ethanol and ethyl acetate extracts were 20.2±15.91 % and 25.5±11.35 % lower than control and the differences were not significant (Figure 3.10 (b)).

3. Bo-Gu-Cao formulation

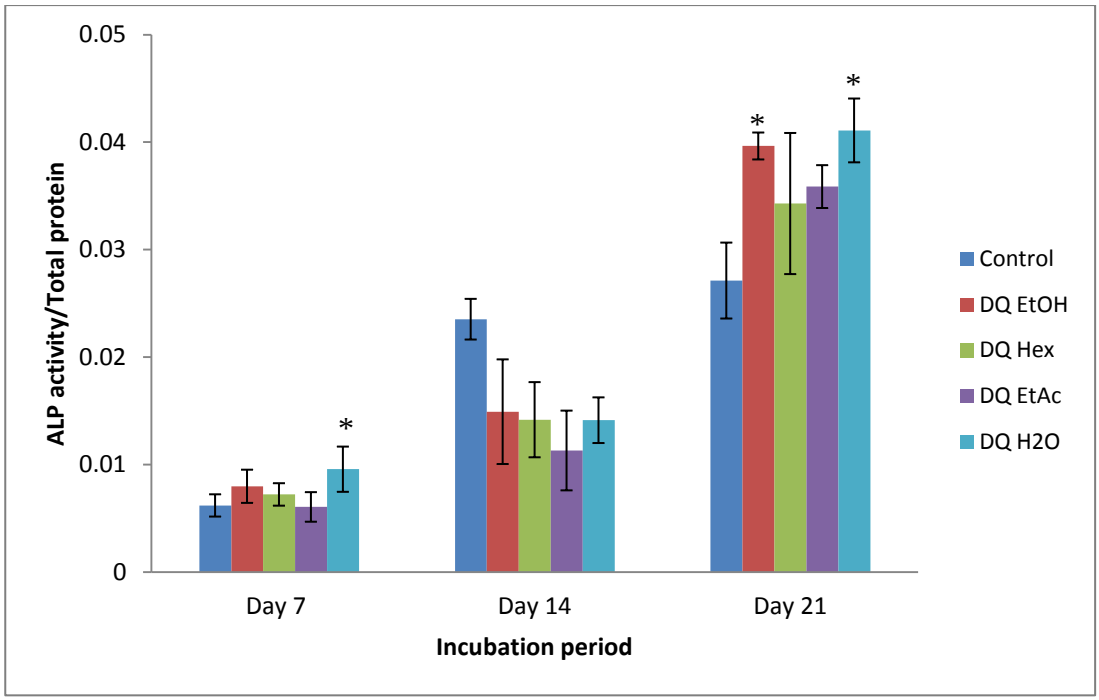
Generally, Bo-Gu-Cao formulation extracts did not enhance ALP activity of rBMS derived osteoblasts after 7 days incubation. Ethanol, hexane and ethyl acetate extracts showed significant downregulation on ALP activity. Hexane showed highest downregulation by 67.2±8.46 %, followed by ethanol (52.5±12.06 %) and ethyl acetate (51.1±18.09 %) extracts. Water extract downregulated ALP activity by 24.5±24.30 % compared to control but the downregulation was not significant.

ALP activity of rBMS derived osteoblasts after 14 days incubation with Bo-Gu-Cao formulation extracts was not remarkable as the result was agreed to 7 days incubation, in which the cells incubated with herbal extracts exhibits downregulation in ALP activity compared to control. Hexane and ethyl acetate extracts downregulated the ALP activity by similar extent. The ALP activity was 51.8±11.45 % lower than control for hexane extract and 51.9±6.64 % lower than control for ethyl acetate extract. Ethanolic extract downregulated ALP activity by 45.6±4.44 %. Downregulationon ALP activity exhibited by rBMS derived osteoblasts incubated with ethanol, hexane and ethyl acetate extracts was statistically significant. Water extract downregulated ALP activity of rBMS derived osteoblasts by 16.3±11.09 % and it was not significant.

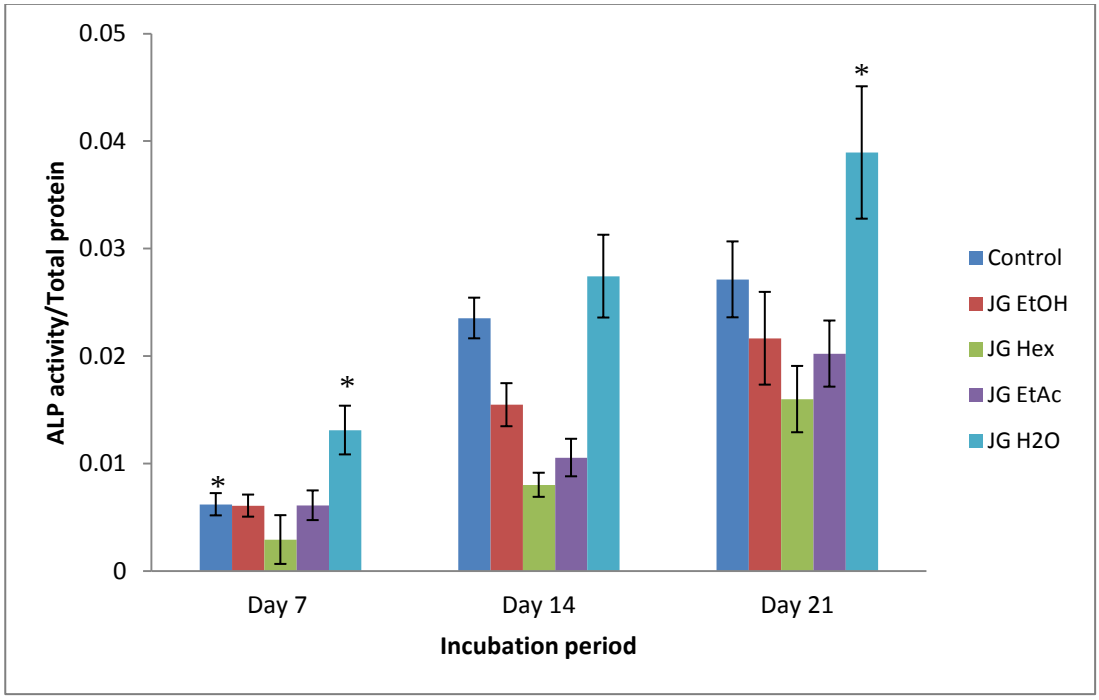
There was something different from 7 and 14 days incubation exhibited in ALP activity of rBMS derived osteoblasts incubated with Bo-Gu-Cao formulation extract. Water extract showed 42.4±10.46 % higher ALP activity than control. The enhancement was

significant. The other 3 extracts showed no enhancement on ALP activity of rBMS derived osteoblasts. Hexane extract downregulated ALP activity significantly by 42.3±16.67 %. The downregulation of ethanol and ethyl acetate extracts was not significant. The downregulation was 34.6±18.83 % and 15.0±46.85 % respectively (Figure 3.10 (c)).

(a)



(b)



(c)

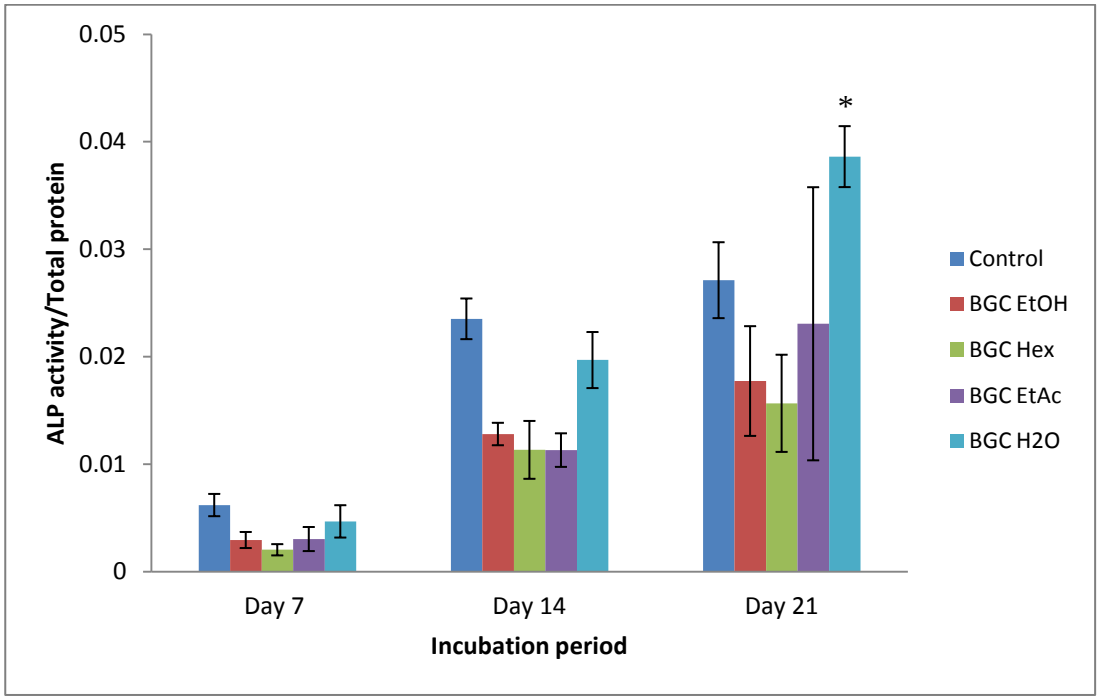
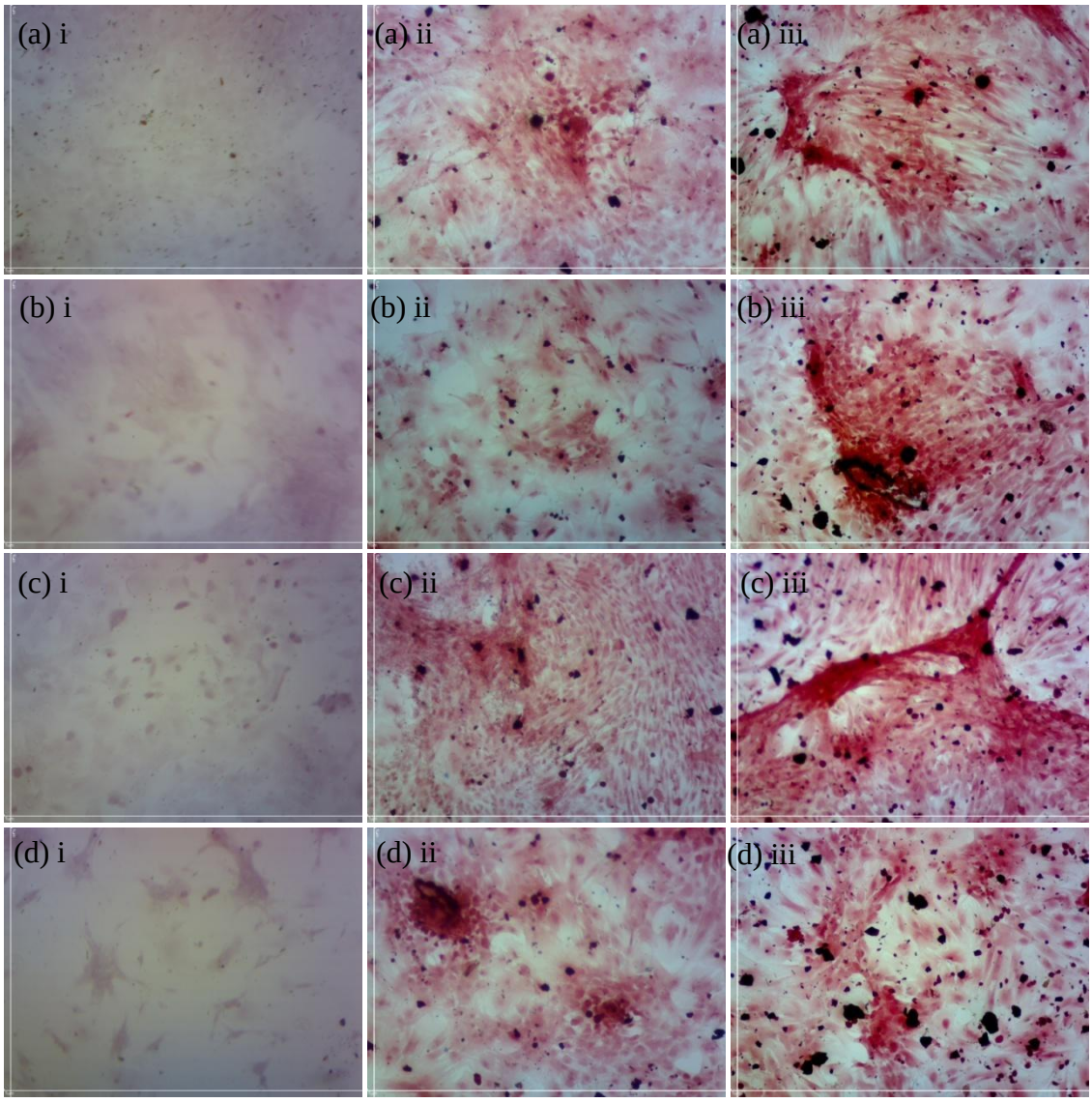


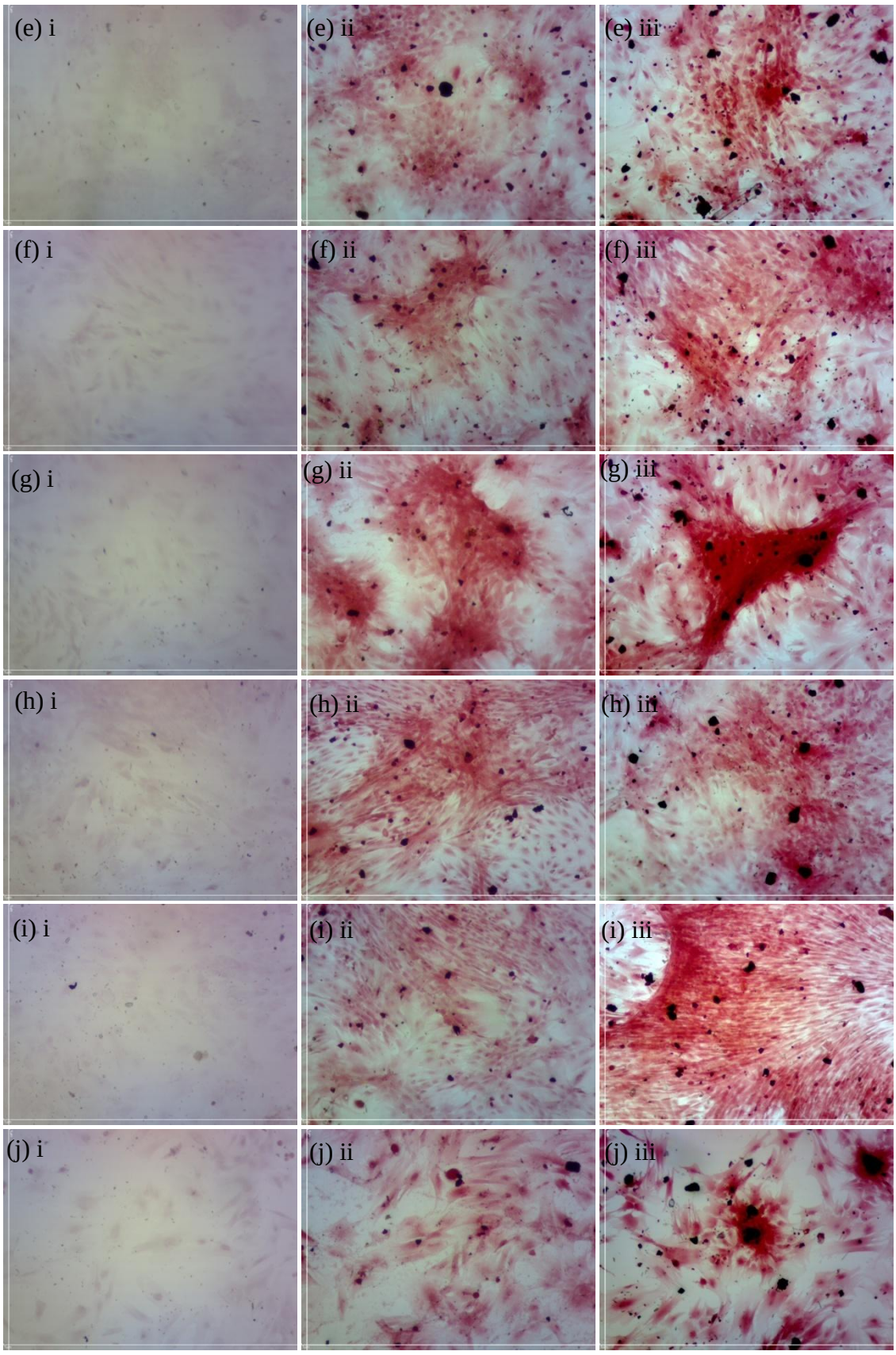
Figure 3.10: ALP activity of rBMS derived osteoblasts after incubated with herbal extracts for 7, 14 and 21 days is shown. Control represents ALP activity/total protein of rBMS derived osteoblasts incubated with control solution in osteogenic medium. Values shown are Mean ALP activity (conversion of one ml of pNPP substrate to p-nitrophenol in one minute)/Total protein ± SD (n=6); *: $p < 0.05$ enhancement over control. DQ EtOH = *D.quercifolia* ethanolic extract, DQ Hex = *D.quercifolia* hexane extract, DQ EtAc = *D.quercifolia* ethyl acetate extract, DQ H_2O = *D.quercifolia* water extract, JG EtOH = *J.gendarussa* ethanolic extract, JG Hex = *J.gendarussa* hexane extract, JG EtAc = *J.gendarussa* ethyl acetate extract, JG H_2O = *J.gendarussa* water extract, BGC EtOH = Bo-Gu-Cao formulation ethanolic extract, BGC Hex = Bo-Gu-Cao formulation hexane extract, BGC EtAc = Bo-Gu-Cao formulation ethyl acetate extract and BGC H_2O = Bo-Gu-Cao formulation water extract. Control value for 7 days incubation = 0.006195±0.001036 µmol/ml/min/µg, control value for 14 days incubation = 0.023525±0.001892 µmol/ml/min/µg, control value for 21 days incubation = 0.027122±0.003533 µmol/ml/min/µg.

3.3.5 Alizarin Red S Staining for calcium deposition

Alizarin red S staining was done for visualization of calcium deposition by rBMS derived osteoblasts under influence of *D.quercilfolia*, *J.gendarussa* and Bo-Gu-Cao formulation extracts. Generally, rBMS derived osteoblasts were stained light red after 7 days of incubation. Little difference could be observed across the cells incubated with different type of herbal extract. The rBMS derived osteoblasts incubated with herbal extracts for 14 days were stained with intenser red color. This could depict calcium deposition was much greater in the cells after 14 days incubation. Generally the cells

incubated for 21 days were stained with slightly redder color compared to cells incubated for 14 days. The alizarin red S staining is a qualitative measure in present study. The microscopic observation of stained cells could not tell the exact difference in calcium deposition between cells incubated with different incubation period or different herbal extracts. However, the photographs give a rough idea of osteoblastic behavior of the cells towards incubation with different type of herbal extract and also confirm calcium deposition by the cells in present study (Figure 3.11).




(e) i
(e) ii
(e) iii
(f) i
(f) ii
(f) iii
(g) i
(g) ii
(g) iii
(h) i
(h) ii
(h) iii
(i) i
(i) ii
(i) iii
(j) i
(j) ii
(j) iii

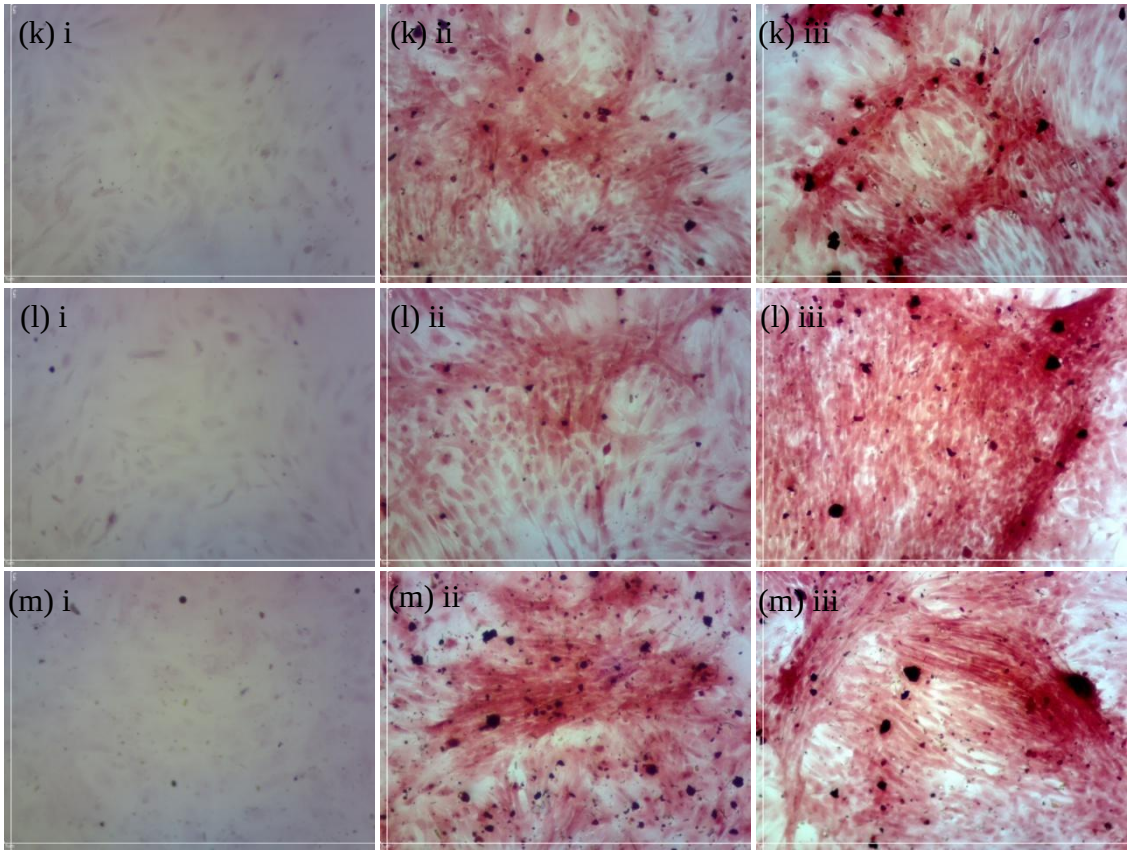


Figure 3.11: Photographs of rBMS derived osteoblasts stained with alizarin red S staining method. The calcium deposit was stained red in the photographs. Redder in color depicts greater calcium deposition by the cells. (a) i, ii, iii = cells incubated 7, 14 and 21 days with control solution, (b) i, ii, iii = cells incubated 7, 14 and 21 days with *D.quercifolia* ethanolic extract, (c) i, ii, iii = cells incubated 7, 14 and 21 days with *D.quercifolia* hexane extract, (d) i, ii, iii = cells incubated 7, 14 and 21 days with *D.quercifolia* ethyl acetate extract, (e) i, ii, iii = cells incubated 7, 14 and 21 days with *D.quercifolia* water extract, (f) i, ii, iii = cells incubated 7, 14 and 21 days with *J.gendarussa* ethanolic extract, (g) i, ii, iii = cells incubated 7, 14 and 21 days with *J.gendarussa* hexane extract, (h) i, ii, iii = cells incubated 7, 14 and 21 days with *J.gendarussa* ethyl acetate extract, (i) i, ii, iii = cells incubated 7, 14 and 21 days with *J.gendarussa* water extract, (b) i, ii, iii = cells incubated 7, 14 and 21 days with Bo-Gu-Cao formulation ethanolic extract, (c) i, ii, iii = cells incubated 7, 14 and 21 days with Bo-Gu-Cao formulation hexane extract, (d) i, ii, iii = cells incubated 7, 14 and 21 days with Bo-Gu-Cao formulation ethyl acetate extract, (e) i, ii, iii = cells incubated 7, 14 and 21 days with Bo-Gu-Cao formulation water extract; inverted microscope, 40X magnification.

3.3.6 Selection of potential herbal extract for dose dependency evaluation

Proliferation and ALP activity were the two main parameters measured in the screening of the effect herbal extracts on rBMS derived osteoblasts. Herbal extract which enhanced both proliferation and ALP activity in rBMS derived osteoblasts were considered

to be potential in bone fracture healing and worth to be studied in dose dependency evaluation to find out the optimum dosage to be used in bone healing.

From the screening result, water extract of *D.quercifolia* induced significant enhancement on rBMS derived osteoblasts proliferation on day 7 and satisfactory but non-significant enhancement on day 14. The extract induced remarkable and significant upregulation of ALP activity on day 21. Hexane extract of *D.quercifolia* induced highest enhancement in the cell proliferation across the incubation period but the enhancement in ALP activity was not significant even incubated for 21 days (Figure 3.8 (a) and 3.10 (a)).

rBMS derived osteoblasts responded similarly to herbal extracts of *J.gendarussa* and Bo-Gu-Cao formulation in terms of cell proliferation and ALP activity. Water extracts of both studied entities induced significant enhancement on proliferation on day 7 and day 14 as well as ALP activity upregulation on and day 21 (Figure 3.8 (b), (c) and figure 3.10 (b), (c)). As told by the herbalist who provided Bo-Gu-Cao formulation which the main constituent of Bo-Gu-Can formulation is *J.gendarussa*, a conclusion can be drawn that *J.gendarussa* was actually responsible for the response of rBMS derived osteoblasts in Bo-Gu-Cao formulation.

Thus, water extracts of *D.quercifolia* and *J.gendarussa* were potential to be further investigated for dose dependency evaluation.

3.4 DOSE DEPENDENCY EFFECT OF HERBAL EXTRACT ON rBMS DERIVED OSTEOBLASTS GROWTH AND DIFFERENTIATION

The herbal extracts were chosen for dose dependency effect based on the result in screening of effect of the herbal extracts on rBMS derived osteoblasts in section 3.3. Water extracts of *D.quercifolia* and *J.gendarussa* were reconstituted with osteogenic medium at different concentration. The final concentrations of the herbal extracts in culture medium were 0, 10, 50, 150, 250, and 500 µg/ml. The effect of the herbal extracts was evaluated when compared to control (cells incubated with 0 µg/ml).

3.4.1 Proliferation rate of rBMS derived osteoblasts after incubation with various concentration of *D.quercifolia* and *J.gendarussa* water extract

Proliferation of rBMS derived osteoblasts was evaluated at day 3, 7, 14 and 21 after incubation with water extracts of *D.quercifolia* and *J.gendarussa* using CCK-8 kit solution. Comparison was made on the cell number between rBMS derived osteoblasts incubated with *D.quercifolia*/*J.gendarussa* water extracts at 10 – 500 µg/ml and control (0 µg/ml).

1. ***D.quercifolia* water extract**

Generally, proliferation of the cells increased from day 3 to day 21, as can be observed in figure 3.12 (a).

At day 3, no significant enhancement can be seen in the cells proliferation from 10 – 500 µg/ml. Notable suppression of proliferation observed on cells incubated with 500 µg/ml of *D.quercifolia*. The suppression was significant (20.1 ± 5.24 %).

After 7 days incubation, proliferation of rBMS derived osteoblasts obviously increased. Significant enhancement was observed at 10 – 150 µg/ml. From the graph, the enhancement of the proliferation started at 10 µg/ml, touched the peak at 50 µg/ml. The

cells incubated with this concentration proliferated 46.9±14.83 % more than control. The proliferation of cells incubated with 150 – 500 µg/ml was less than 50 µg/ml and went down from 150 – 500 µg/ml. Cells incubated with 500 µg/ml proliferated lower than control (-8.3±10.69 %) but the difference was not significant.

On day 14, significant enhancement of cells proliferation can be seen from 10 µg/ml to 250 µg/ml. The proliferation of cells incubated with 10 µg/ml was 39.6±8.24 % more than control. The proliferation rate went up at 50 µg/ml, which was 45.7±5.97 % more than control. The cells proliferation under effect of 150 µg/ml recorded highest enhancement with 48.2±16.40 % more than control. Proliferation of the cells went down from 250 µg/ml which was 29.1±14.98 % more than control. Cells incubated with 500 µg/ml were again proliferated less than control, but the suppression was not significant (-10.4±7.01 %).

On day 21, proliferation of control cells was almost the same as on day 14. The proliferation of cells incubated with 10 µg/ml was lower than on day 14. The proliferation of cells increased from 10 µg/ml to 150 µg/ml. The cells incubated with 150 µg/ml showed highest enhancement compared to control cells (71.4±20.03 %) The cells proliferation incubated with 250 µg/ml was lower than 150µg/ml. However, the cells incubated with 10 – 250 µg/ml showed significant enhancement over control cells. The cell proliferation under incubation of 500 µg/ml was almost the same as day 14, which was 9.6±13.56 % lower than control. The suppression was not significant.

2. *J.gendarussa* water extract

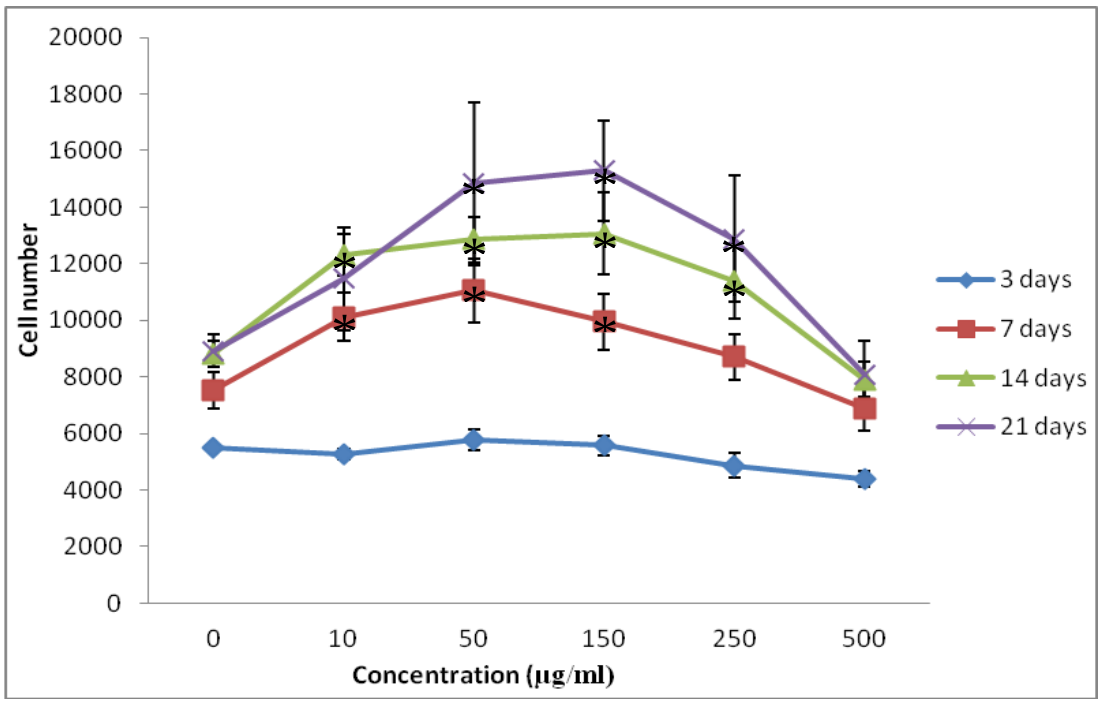
On day 3, proliferation of rBMS derived osteoblasts incubated with 0 – 500 µg/ml showed no significant difference. Only cells incubated with 500 µg/ml proliferated lower than control.

On day 7, proliferation of the cells incubated with 10 – 500 µg/ml of *J.gendarussa* water extract showed significant enhancement over control. 10 µg/ml induced highest enhancement, which was 30.7±11.31 % higher than control. However, the enhancements by other concentrations was not much different from 10 µg/ml, which were 25.9±9.69 % (50 µg/ml), 27.0±7.78 % (150 µg/ml), 26.3±5.13 % (250 µg/ml) and 21.3±4.61 % (500 µg/ml). Concentrations played little part in enhancement of the proliferation of the cells for day 7 incubation. A plateau can be seen on the graph of proliferation versus concentrations on day 7.

rBMS derived osteoblasts incubated with 10 µg/ml was again showed highest enhancement over control on day 14. The enhancement was 35.3±6.07 % over control and the enhancement was significant. The proliferation of cells incubated with 50 – 500 µg/ml was lower than 10 µg/ml, and showed enhancement over control though. Enhancement by 50 – 250 µg/ml was significant but not significant by 500 µg/ml.

On day 21, the proliferation of rBMS derived osteoblasts incubated with 10 – 500 µg/ml showed enhancement over control at various extents. The enhancements were 32.5±18.60 % (10 µg/ml), 34.8±23.19 % (50 µg/ml), 29.6±27.08 % (150 µg/ml), 34.7±33.23 % (250 µg/ml) and 2.9±15.61 % (500 µg/ml). However, the enhancements were not significant.

(a)



(b)

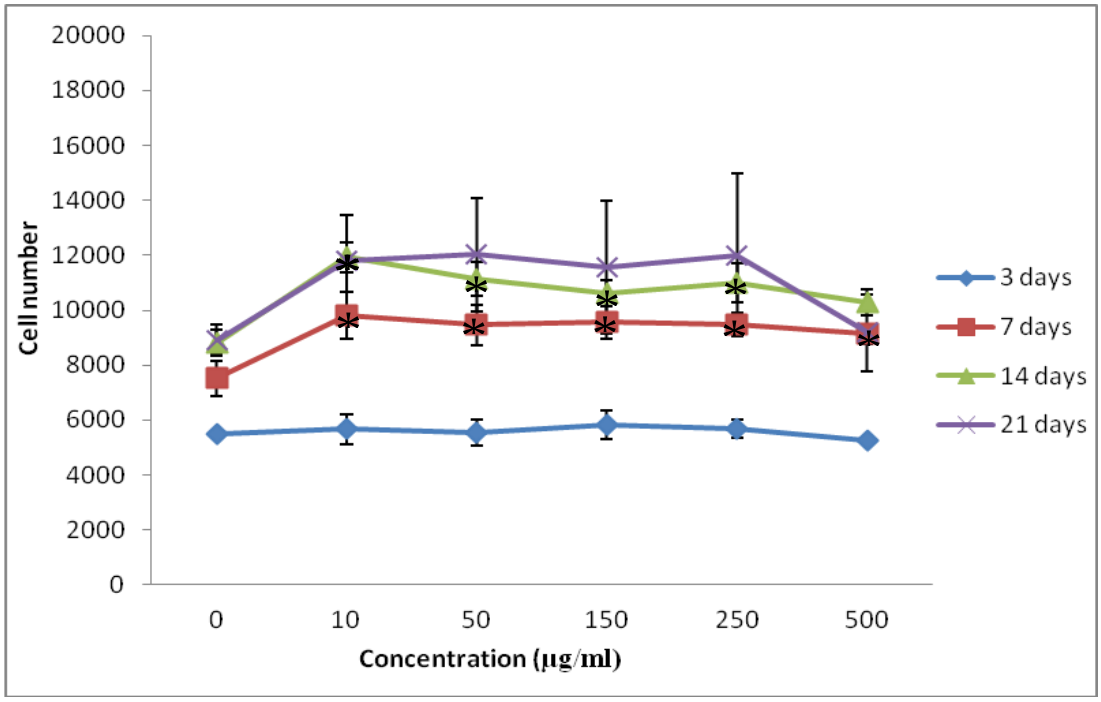


Figure 3.12: Proliferation of rBMS derived osteoblasts after incubated with (a) *D.quercifolia* and (b) *J.gendarussa* for 3, 7, 14 and 21 days is shown. Control represents rBMS derived osteoblasts incubated with 0 µg/ml of herbal extracts. Values shown are Mean cell number ± SD (n=6); *: $p<0.05$ enhancement over control. (a) 0 = *D.quercifolia* water extract 0 µg/ml, 0 = *D.quercifolia* water extract 10 µg/ml, 50 = *D.quercifolia* water extract 50 µg/ml, 150 = *D.quercifolia* water extract 150 µg/ml, 250 = *D.quercifolia* water extract 250 µg/ml, 500 = *D.quercifolia* water extract 500 µg/ml, (b) 0 = *J.gendarussa* water extract 0 µg/ml, 0 = *J.gendarussa* water extract 10 µg/ml, 50 = *J.gendarussa* water extract 50 µg/ml, 150 = *J.gendarussa* water extract 150 µg/ml, 250 = *J.gendarussa* water extract 250 µg/ml, 500 = *J.gendarussa* water extract 500 µg/ml. Control value for 3 days incubation = 5485 ± 97 cell/well, control value for 7 days incubation = 7522 ± 631

cell/well, control value for 14 days incubation = 8820 ± 451 cell/well, control value for 21 days incubation = 8916 ± 582 cell/well.

3.4.2 ALP detection for rBMS derived osteoblasts after incubation with various concentration of *D.quercifolia* and *J.gendarussa* water extract

ALP activity of rBMS derived osteoblasts was evaluated at day 7, 14 and 21 after incubation with water extracts of *D.quercifolia* and *J.gendarussa*. The result was then normalized to total protein content of the cells. Comparison was made on the ALP activity/total protein content between rBMS derived osteoblasts incubated with *D.quercifolia*/*J.gendarussa* water extracts at 10 – 500 µg/ml and control (0 µg/ml).

1. ***D.quercifolia* water extract**

On day 7 evaluation, ALP activity of rBMS derived osteoblasts incubated with 10 – 500 µg/ml *D.quercifolia* water extract did not show significant difference when compared to control. Cells incubated with 250 µg/ml showed highest enhancement, which was 33.3±27.73%. ALP activity of cells incubated with 10 µg/ml was enhanced as much as 19.8±41.61 %, which was the second highest among the *D.quercifolia* water extract concentrations. Cells incubated with 500 µg/ml were enhanced lowest among the 5 concentrations (6.6±33.42 %).

On day 14, contrary result to day 7 was shown. ALP activity of cells incubated with 250 µg/ml had shown downregulation compared to control (-7.6±15.61 %). It was the only concentration that caused dowregulation to ALP activity among the 5 concentrations. Cells incubated with 50 µg/ml upregulated ALP activity the most (28.4±16.75 %), closely followed by 150 µg/ml (25.2±33.42 %). The cells incubated with 10 µg/ml had shown fewer enhancements (13.2±17.22 %). The ALP activity of cells incubated with 500 µg/ml

was only 1.9±8.33 % more than control. The difference between the ALP activity of 10 – 500 µg/ml and control was not significant.

The ALP activity evaluated on day 21 was similar to the result of day 14 but with higher enhancement. Cells incubated with 250 µg/ml was again the only concentration which showed downregulation in ALP activity (-3.56±12.49 %). The cells incubated with 50 µg/ml were the only studied individuals among the 5 concentrations that showed significant enhancement in ALP activity. The enhancement percentage was 40.2±10.44 % over control. The ALP activity of cells incubated with 150 µg/ml showed second highest enhancement over control (34.4±22.69 %), followed by 10 µg/ml (19.7±15.61 %) and 500 µg/ml (16.3±20.17 %) (Figure 3.13 (b)).

2. *J.gendarussa* water extract

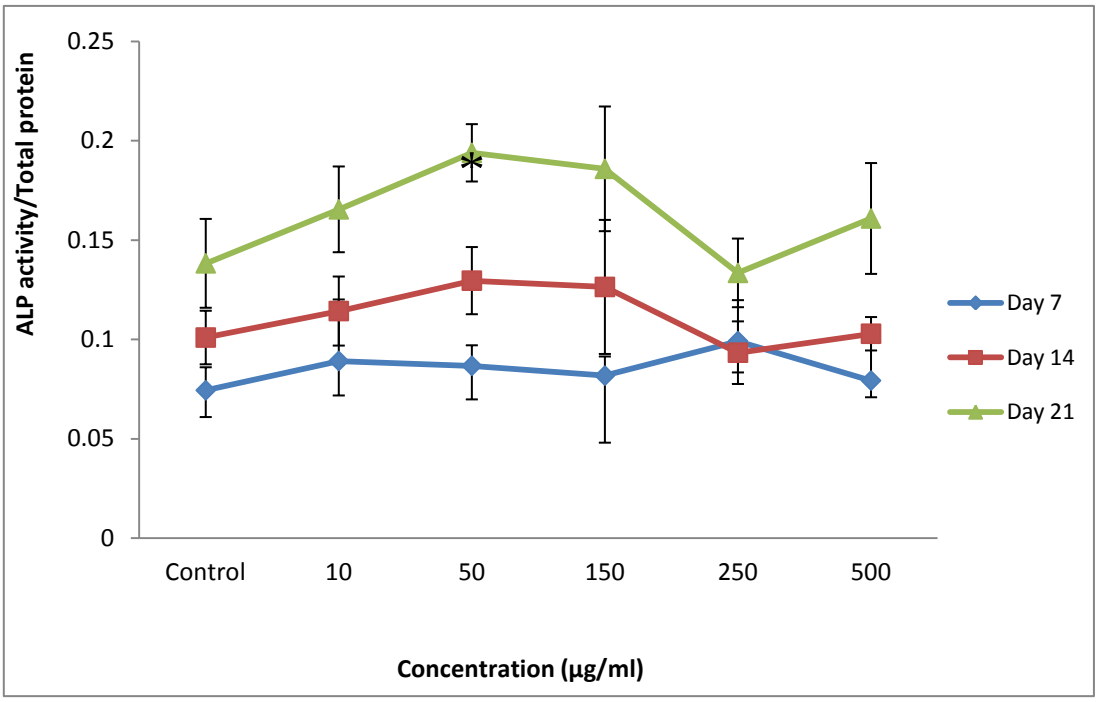
After 7 days incubation with *J.gendarussa* water extract (0 – 500 µg/ml), the ALP activity of rBMS derived osteoblasts was almost the same. There is no significant difference shown.

On day 14, there is no significant difference between the ALP activity of rBMS derived osteoblasts incubated with 0 – 50 µg/ml. Interestingly, the ALP activity of cells incubated with 150 µg/ml and 250 µg/ml had shown relatively great enhancement over control. The cells incubated with the 2 concentrations showed 75.7±17.15 % and 75.3±24.57 % enhancement over control respectively. The ALP activity of cells incubated with 500 µg/ml was lower than the former two, which was 43.7±14.50 % more than control. The enhancement of ALP activity showed by cells incubated with 150, 250 and 500 µg/ml was significant.

On day 21, the ALP activity of rBMS derived osteoblasts incubated with 10 – 500 µg/ml was upregulated and showed enhancement over control. Similar to result in day 14,

enhancement by 10 and 50 µg/ml was not significant. On the other hand, the enhancement by 150, 250 and 500 µg/ml was significant. However, the enhancement percentage shown in 150 and 250 µg/ml was lower than day 14, which was 42.3±6.02 % and 45.5±19.26 % respectively. The ALP activity of cells incubated with 500 µg/ml was 42.2±36.36 % (Figure 3.13 (b)).

(a)



(b)

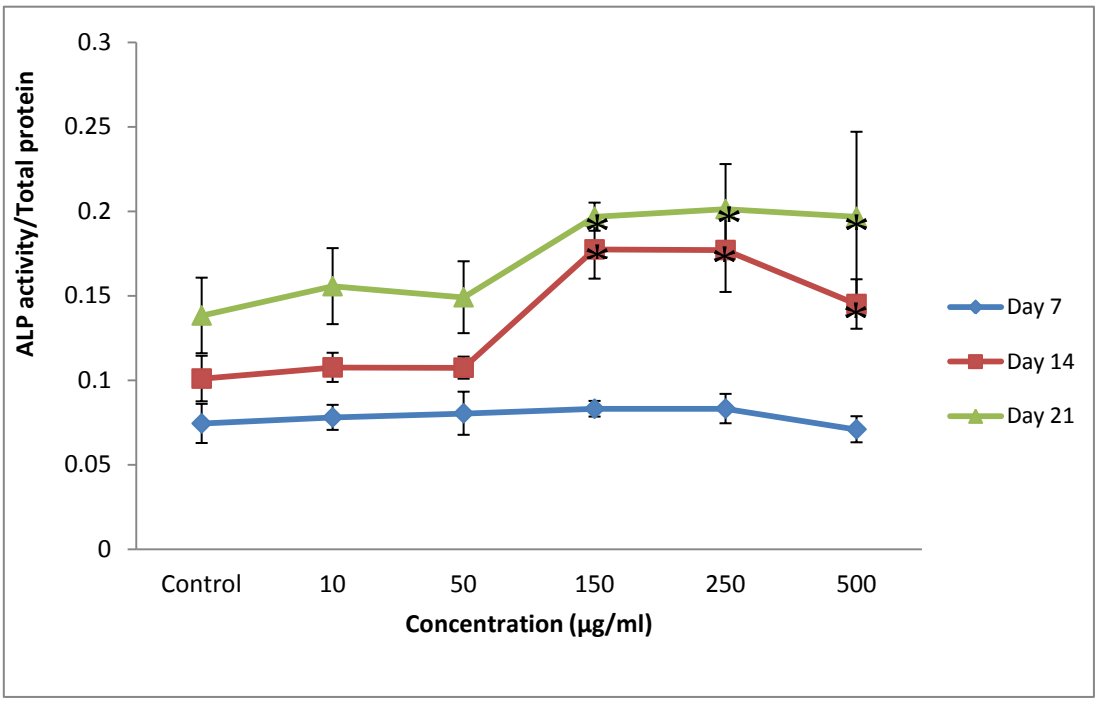


Figure 3.13: ALP activity normalized to total protein of rBMS derived osteoblasts after incubated with (a) *D.quercifolia* and (b) *J.gendarussa* for 7, 14 and 21 days is shown. Control represents ALP activity/total protein of rBMS derived osteoblasts incubated with 0 µg/ml of herbal extracts. Values shown are Mean ALP activity (conversion of one ml of pNPP substrate to p-nitrophenol in one minute)/Total protein ± SD (n=6); *: $p<0.05$ enhancement over control. (a) 0 = *D.quercifolia* water extract 0 µg/ml, 0 = *D.quercifolia* water extract 10 µg/ml, 50 = *D.quercifolia* water extract 50 µg/ml, 150 = *D.quercifolia* water extract 150 µg/ml, 250 = *D.quercifolia* water extract 250 µg/ml, 500 = *D.quercifolia* water extract 500 µg/ml, (b) 0 = *J.gendarussa* water extract 0 µg/ml, 0 = *J.gendarussa* water extract 10 µg/ml, 50 = *J.gendarussa* water extract 50 µg/ml, 150 = *J.gendarussa* water extract 150 µg/ml, 250 = *J.gendarussa* water extract 250 µg/ml, 500 = *J.gendarussa* water extract 500 µg/ml. Control value for 7 days incubation = 0.074383±0.011588 µmol/ml/min/µg, control value for 14 days incubation = 0.100942±0.013470 µmol/ml/min/µg, control value for 21 days incubation = 0.138278± 0.022360 µmol/ml/min/µg.

CHAPTER 4:

DISCUSSIONS

4.1 ISOLATION AND CULTURE OF RBMSC AND ITS OSTEOGENICITY

Culture of stem cells has drawn vast interest from biological and medical fields. Applications of stem cells in certain disease management has been proven to be potential (e.g. cancers, cardiovascular disorders, diabetes, muscular dystrophy, wound healing (Ott *et al.*, 2005; Price *et al.*, 2007; Sagar *et al.*, 2007; Branski *et al.*, 2009; Vija *et al.*, 2009; Shah, 2012)). Thus stem cell is a good research model in tissue engineering study. Overall the researches focus on evaluating stem cell response towards certain stimulant or utilizing them in certain application. In present study, BMSC isolated from rat was used.

4.1.1 rBMSC as a research model

Stem cell can be obtained from various sources, including adipose tissue, bone marrow, embryonic tissue and blood circulation. From the options listed, bone marrow isolated from rat was chosen to be used in this study. Apart from the pluripotency and self renewable ability which shared with other types of stem cell, there are several reasons rBMSC was chosen.

First of all, quantity of stem cell obtained from bone marrow is considered more than sufficient. In the present study, rBMSC obtained from each SD rat (6 – 8 week old, 150 – 170g, only bone marrow from tibiae and femora of both hind legs was used) were isolated and nurtured in a T-75 culture flask. Bone marrow only consists of less than 0.01% of mesenchymal stem cells (BMSC), and the percentage drops in accordance to the age of the host. Thus in this study, young adult Sprague-Dawley (SD) rats were sacrificed to obtained rBMSC. rBMSC obtained would actively proliferate and differentiate, and increase the success rate of obtaining rBMSC culture as well as achieving faster confluency in shorter period. The bone marrow must be harvested as fast as possible as a longer time

would reduce the viability of these rBMSC. Usually the cells reached confluency after 1 – 2 weeks incubation and there would be 1 – 2 million cells in the flask. Normally confluent cells in one flask could be split into two or three flasks in each passage. Cells at passage 2 – 6 were harvested for cell seeding in certain assays.

Secondly, rBMSC were readily available source in this study. SD rat were supplied by Animal House at University Malaya Medical Center. Occasionally they couldn't provide rat because they ran out of suitable rat (age and weight were not fit to requirements) but rats were always available again within one or two weeks afterwards. As compared to human bone marrow, rat bone marrow can be acquired more often. Availability of human bone marrow depends on patient status of collaborated hospital. A continuous readily available source of research model is important in a study as to let the study being carried out smoothly.

Furthermore, rBMSC have been identified as a multipotent cell, similar to human BMSC, in terms of being used in tissue engineering research.

4.1.2 Culture of rBMSC

Prior to rBMSC isolation process, rat was immersed in sacrificed and 70% alcohol for 5 – 10 minutes to avoid contamination after cervical dislocation. The bone marrow must be harvested as quickly as possible to maximize viability of these rBMSC. Isolated bone marrows were plated into culture flask using primary medium and incubated with 5% CO_2, relative humidity of 95%, at 37 ^{0}C. Culture medium containing appropriate amount of antibiotics/antimycotic (1% of antibiotic – antimycotic solution was use in this study) was prepared to prevent growth of contaminants such as bacteria and moulds, as well to provide nutrient for rBMSC growth. Culture of these rBMSC should be in condition of 5% CO_2, 37

^{0}C and relative humidity of 95%. The conditions are to simulate *in vivo* environments as best as possible. Culture medium with phenol red was used in this study. Change of culture medium color could indicate contamination, together with turbidity in the culture medium. Usually fast random moving objects (which could indicate bacterial contamination) or stay still polygonal objects (which could indicate fungal infection) can be observed under microscope. Such contamination could happen during isolation process or during passaging, changing medium and so on. Immediate disposal of these contaminated cultures from incubator is required to avoid spreading of these contaminants to uncontaminated cultures. Isolated bone marrows were cultured for three to four days in primary medium to allow attachment of rBMSC onto the bottom surface of culture flask before change of medium for the very first time. Changing of culture medium must be done gently. Harsh movement during changing medium could cause detachment of rBMSC from the flask. The unattached cells ought to be removed by repeated washing repeatedly with PBS, as these cells or unwanted cells may be eventually die and attach firmly on the bottom surface of the culture flask, thus limiting rBMSC growth, and may cause death to the rBMSC.

rBMSC cultured in primary medium obtained in this study had apparent nuclei, were fibroblastic in shape and attached and spread onto the bottom surface of culture flask.

4.1.3 Characterization of rBMSC

Characterization of rBMSC can be done in several ways. Basically the key features of rBMSC are fibroblastic appearance, adherence to plastic (culture flask) surface, multipotent (when induced by certain growth factor *in vitro*) and identification of particular cell surface markers.

In this study, adherence to plastic surface could always be observed under microscope within several hours of incubation in CO_2 incubator after isolation. The characteristic can be seen more clearly after changing medium and removing all unattached cell. The cells appeared to be fibroblastic after few days of incubation. Cytoplasm tended to be elongated and appeared in spindled shaped.

The confirmation of rBMSC cell surface markers on the cells used in the present study is crucial. Many cell surface markers can be determined on rBMSC. SH2 and SH3 which correspond to CD105 and CD73 respectively were the two very first found markers in the study of Pittenger *et al.,* 1999. CD is the standard nomenclature for cell surface molecules. It stands for "cluster of differentiation". In Dominici *et al.,* 2006, CD90 was found positively expressed together with the former two on BMSC. The cell surface markers which should be negatively expressed are CD11b or CD14, CD19 or CD79a, CD 31 or PECAM-1, CD34, CD45, and HLA-DR. The negative expressed markers are used to exclude hematopoietic cells, which present during isolation process and could be cultured together with rBMSC. There are several methods to identify rBMSC cell surface markers, while fluorescence-activated cell sorting method was employed in this study using flow cytometry. As can be seen in the result, a cell population of CD31-, CD45- and CD90+ was found in the rBMSC cultures. This result was enough to confirm successful of rBMSC culture. However, there was one more cell population with CD45+ found in the culture. This could indicate the contamination of hematopoietic cells in the culture, and this probably due to using rBMSC at low passage.

Multipotent characteristic of rBMSC was determined. Three lineage differentiation method (adipogenesis, osteogenesis and chondrogenesis) was commonly employed to assess multipotency of stem cell. Commercial available differentiation kit was used in the

present study. The advantage of using commercial available kit is the preparation of the growth factor is proven to be working on inducing BMSC differentiation. The rBMSC cultured with respective differentiation kit solution were stained with respective staining method. Certain characteristics were to be found out to confirm successful differentiation. Discovery of lipid droplet was to confirm adipogenesis, discovery of mineralization was to confirm osteogenesis, and discovery of cartilage formation was to confirm chondrogenesis. Staining methods for respective mature cell characteristics was done in the present study and the qualitative methods were enough to prove the differentiation. Differentiation of rBMSC to adipocyte and osteoblast was clearly identified after staining. Staining of cartilage in chondrogenic differentiation was very mild. This could be due to difficulties in cell seeding. Method of high cell seeding number in droplets was used (recommended by differentiation kit solution supplier). There is another cell seeding method that could enhance the differentiation which is high density pellet culture (Bosnakovski *et al.*, 2004).

4.1.4 Osteogenicity of rBMSC

rBMSC has been proven to be able to differentiate into osteoblast under stimulation of proper growth factor. In present study, dexamethasone, β-glycerophosphate and L-ascorbic acid were added to primary medium to be used as osteogenic medium. There are reasons the three components are added to primary medium to make an osteogenic medium and there were studies that playing around with the concentration of the respective growth factors mentioned above to determine the optimum condition for osteogenic induction on rBMSC. The concentrations of dexamethasone, β-glycerophosphate and L-ascorbic acid were 10 nM, 5 mM and 50 μg/ml respectively (suggested by Chai *et al.*, 2006).

According to Maniatopoulos, 1988, replacing primary medium with osteogenic medium 4 days after cell isolation would result in optimum osteogenic growth of rBMSC. When these rBMSC were further cultured in osteogenic medium for 2 weeks, rounded and spindled newly formed rBMSC were observed on top of the confluent single cells layers. Trypsinization and subculture of these rBMSC in osteogenic medium, gave rise to better homogenicity of BMS-derived osteoblasts which were described as rounded or cuboidal cells with dense and apparent nuclei, and multiple cytoplasm possesses. Observation of extracellular matrix and mineral nodule deposition within the cultures were evident of differentiation of rBMSC into BMS-derived osteoblasts. However, the microscopic observation alone was not enough and Alizarin Red S staining showed calcium deposition can further confirm the osteogenesis of rBMSC cultured with osteogenic medium.

4.2 BIOCHEMICAL ENHANCER IN MEDICINAL HERBS

In the present study, hypothetically biochemical enhancers (active compounds/working compounds) for bone regeneration could be found from the medicinal herbs. Preparation of herbal extract involved solvent extraction. Four types of solvent were used (ethanol, hexane, ethyl acetate and water). Theoretically, ethanol would extract all compounds from a medicinal herb. Partial isolation was done on ethanolic extract using hexane, ethyl acetate and water and resulted in obtaining hexane extract (non-polar compound), ethyl acetate extract (semi polar compound) and water extract (polar compound). Herbal extracts were then reconstituted in culture medium and incubated with rBMS derived osteoblasts directly. Traditionally, the medicinal herbs are prepared by herbalist and applied topically on the fracture site of the patient. The active compounds are expected to diffuse into the fracture bone and induce osteoblast proliferation and differentiation.

Prior to herbal extract preparation, medicinal herbs were taken to a taxonomist and been identified. This was to avoid misunderstanding when looking up to info for the particular medicinal herbs.

Local availability and yield percentage of extract are the two factors needed to be studied carefully other than studying in the biological effect on the subject before the herbal extracts come to commercialized stage. Local availability ensures the herbs need not to be imported from overseas and can be mass planted locally. In this study, the medicinal herbs were obtained from local herbalists and the herbs were planted by the herbalists. High yield percentage ensures high productivity from minimum raw material. Yield percentage of the herbal extracts from the studied medicinal herbs is considered satisfying.

4.3 EFFECT OF HERBAL EXTRACTS ON rBMS DERIVED OSTEOBLASTS

A total of 12 herbal extracts was screened for effects on rBMS derived osteoblasts. Basically, proliferation rate and ALP activity were determined. Alizarin Red S staining was done only for confirmation of calcium deposition after incubation with herbal extracts. Two herbal extracts were chosen to carry on with dose dependency effect, and proliferation rate, ALP activity and osteocalcin production were assessed after incubation with different concentrations of the herbal extracts.

Proliferation rate was determined by CCK-8 Kit solution. There are advantages of using the kit solution. According to the product information provided by the supplier the kit solution gives higher sensitivity to the mitochondrial activity compared to other colorimetric cell proliferation assays such as MTT and alamar blue. The preparation step and time for this kit solution is minimal because it is a ready-to-use solution and no mixing step involved before the solution can be used. The solution can be used with phenol red added medium (which was used in present study). However the OD reading of each well should be determined by substracting the OD reading from blank well (which contained culture medium only and no cell). A standard curve of OD versus cell number was plotted. The standard curve showed correlation between OD generated by formazan and cell number. Cell number can be estimated using the standard curve.

ALP activity was determined by conversion rate of pNPP substrate and normalized to total protein. ALP is an enzyme, which is also a protein. Normalizing the ALP activity to total protein can give real changes of ALP production by the rBMS derived osteoblasts after incubation with herbal extracts.

4.3.1 Screening of effect of herbal extracts on rBMS derived osteoblasts

Due to big amount of type of herbal extracts studied in this study, the effect of the herbal extracts had to be screened before the herbal extracts were prepared at various concentrations in dose dependency effect. The herbal extracts were standardized at 100 μg/ml in culture medium.

Generally, rBMS derived osteoblasts incubated with herbal extracts proliferated as much as control after 3 days incubation. There is no significant difference. This could indicate that the herbal extracts need time to cause an effect on the proliferation of the cells.

Hexane extract of *D.quercifolia* induced highest enhancement on proliferation of rBMS derived enhancement on day 7 and day 14. Water extract of *J.gendarussa* and *D.quercifolia* were second and third highest enhancers to rBMS derived osteoblasts proliferation. Both water extracts of *D.quercifolia* and *J.gendarussa* significantly enhanced ALP activity on day 7 and day 21 incubation.

Proliferation rate and ALP activity/total protein are not necessary correlated directly. When cells are focused on proliferation, differentiation activity could be lesser and vise versa.

As mentioned in introduction section, *D.quercifolia* was chosen as a studied object because it was expected to possessed similar active compound as another well-researched bone herb, *D.fortunei* as the two herbs are belonged to same family (Ramesh *et al.*, 2001, Wang *et al*., 2011). In study of Sun *et al.*, 2002, crude extract was used to determine the effect of *D.fortunei*. The crude extract is supposed to be similar to ethanolic extract as ethanolic extract would contain polar and non polar compounds. The ethanolic extract of *D.quercifolia* did show enhancement in ALP activity after 21 days incubation and no enhancement on proliferation rate. This is contrary to the Sun *et al.*, 2002 study as they

found the crude extract of *D.fortunei* increase proliferation from day 1 to day 7 at concentration of 100 μg/ml. Naringin is the main working compound in *D.fortunei* on enhancing osteoblastic behavior of bone cell (Peng-Zhang *et al.*, 2009). It is a polymethoxylated flavonoid. Since it is water soluble compound, it is expected to present in water extract of *D.quercifolia* also. However, lack of enhancement of osteoblastic behavior by ethanolic extract of *D.quercifolia*, a theory can be postulated that active compound in water extract could be suppressed by compounds in crude extract.

Effects of herbal extracts of *J.gendarussa* and Bo-Gu-Cao formulation on rBMS derived osteoblasts are very similar. As told by the herbalist who provided Bo-Gu-Cao formulation, main constituent in Bo-Gu-Cao formulation is *J.gendarussa*. Thus the effects on rBMS derived osteoblasts induced by Bo-Gu-Cao formulation were largely due to *J.gendarussa*. In Chinese traditional medicine, a mixture of herbs does not necessary have only one effect or target. Herbal mixture other than *J.gendarussa* in Bo-Gu-Cao formulation could act differently such as helping in angiogenesis and reducing inflammation (Wang *et al*., 2004, Yoo *et al* ., 2008).

Water extract of *D.quercifolia* and *J.gendarussa* were the best performer in terms of enhancing both proliferation and ALP activity.

4.3.2 Dose dependency effect of water extract of *D.quercifolia* and *J.gendarussa*

Five concentrations from the respective water extracts were prepared, which were 10, 50, 150, 250 and 500 μg/ml.

Proliferation rate at day 3 was expected as there was no significant difference between the water extracts and the control. For water extract of *D.quercifolia*, concentrations of 10, 50 and 150 μg/ml enhanced proliferation rate significantly and

generally proliferation rate was lower at 250 and 500 µg/ml. This indicates that there is dose dependency effect of water extract of *D.quercifolia* on rBMS derived osteoblasts. Any concentration higher than 500 µg/ml is expected to cause lower proliferation rate on rBMS derived osteoblasts and could be toxic to the cell. Enhancement on ALP activity by water extract of *D.quercifolia* started at 10 µg/ml as well. However, on day 14 and day 21, the peak was at 50 µg/ml, and started to decrease at 150 µg/ml. The ALP activity hit lowest at 250 µg/ml compared to other concentrations. From 10-250 µg/ml, their effect on ALP activity of rBMS derived osteoblasts was considered dose dependent.

Proliferation rate induced by water extract of *J.gendarussa* was not really dose dependent. As can be seen from the result, the proliferation rate increased at 10 µg/ml and did not show much difference from 10-500 µg/ml for day 7 – 21 incubation. Proliferation rate at 500 µg/ml on day 21 decreased sharply compared to cell proliferation incubated with other concentrations. This could depict that proliferation rate of osteoblasts can be harmed at high concentration of water extract of *J.gendarussa* for long term incubation. For ALP activity, a trigger dosage can be clearly seen at 150 µg/ml on day 14 and day 21. ALP activity of BMS derived osteoblasts incubated with higher concentrations did not enhanced or downregulated significantly compared to 150 µg/ml. Any concentrations lower than 150 µg/ml did not enhance ALP activity over compared to control.

The results of dose denpendency effect have shown that dosage plays a big role in using medicinal herb. For instance, the dosage higher than optimum dose of water extract of *D.quercifolia* caused downregulation of proliferation as well as differentiation of rBMS-derived osteoblasts. This depicts that the use of dosage higher than optimum dose would not help in bone fracture healing, and there is possibility of slowing the healing process if dose of too high is used. On the other hand, water extract of *J.gendarussa* did not exhibit

dose dependency effect on rBMS-derived osteoblasts. Thus, a threshold dosage is enough to cause desired effect on bone fracture healing. By knowing the threshold dose, the herb is used effectively.

The findings of the present study not only can contribute to provide an alternative to bone fracture management, bone tissue engineering may benefit from these findings as well. For instance, incorporation of medicinal herbs with scaffold is much anticipated. Scaffold is designed and created to aid and enhance bone fracture healing in bone tissue engineering. With the addition of medicinal herbs to scaffold, bone fracture healing is expected to be more effective, causing less pain in patient brought by bone fracture and shorten healing period.

Osteoblasts proliferation and mineralization are mediated by estrogens receptors (Ikeda *et al*., 2011). Although the possible pathway or mechanism of effect of water extracts of *D.quercifolia* and *J.gendarussa* was not examined in the present study, it is believed that the presence of phytoestrogen in the extracts triggered the enhancement effect on proliferation and mineralization of rBMS derived osteoblasts.

CHAPTER 5:

CONCLUSIONS

From the result obtained in this study, rBMSC was able to be isolated and cultured for long term. The microscopic appearance of rBMSC should be fibroblastic and adherent to plastic surface in order to grow continuously *in vitro*. However, the isolated bone marrow contained both hematopoietic cell and stromal cell. It was difficult to eliminate the hematopoietic cell from the bone marrow cultures completely by washing and changing medium method. Result from cell analysis in flow cytometry indicated this. However, homogeneity of the rBMSC can be improved by repeated washing and passaging of the cell. Successfully differentiated to adipocyte, osteoblast and chondrocyte could point to the elimination of hematopoietic cell.

In screening of effect of the herbal extracts on rBMS derived osteoblasts, *D.quercifolia*, *J.gendarussa* and Bo-Gu-Cao formulation were identified to be containing biochemical enhancer for rBMS derived osteoblasts proliferation and differentiation. Hexane extract of *D.quercifolia* and water extracts of *D.quercifolia*, *J.gendarussa* and Bo-Gu-Cao formulation enhanced proliferation of rBMS derived osteoblasts the most during day 7 and day 14. Water extracts of *D.quercifolia* and *J.gendarussa* showed most promising in enhancing ALP activity of rBMS derived osteoblasts. The result of Bo-Gu-Cao formulation was very similar to *J.gendarussa*'s and suspected that the actual working component in Bo-Gu-Cao formulation was actually from *J.gendarussa*.

A second achievement in this project was that dose dependency effect of water extracts of *D.quercifolia* did exist on both proliferation and ALP activity of rBMS derived osteoblasts. The optimum dosage of *D.quercifolia* is between 50 – 150 μg/ml. On the other hand, no dose dependency effect of *J.gendarussa* was found on proliferation and ALP activity of rBMS derived osteoblasts. However, there was a threshold dosage of *J.gendarussa* for proliferation (10 μg/ml) and ALP activity (150 μg/ml).

Further investigation should focus on isolation of biochemical enhancers/active compound/working compound from water extracts of *D.quercifolia* and *J.gendarussa*. The isolated compound/s should be tested both *in vitro* and *in vivo*.

REFERENCES

Abebe F, Erko B, Gemetchu T and Gundersen S G (2005). Control of *Biomphalaria pfeifferi* population and schistosomiasis transmission in Ethiopia using the soap berry endod (*Phytolacca dodecandra*), with special emphasis on application methods. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **99**: 787-794.

Alcock J, Scotting P and Sottile V (2007). Bergmann glia as putative stem cells of the mature cerebellum. *Medical Hypotheses* **69**: 341-345.

Alexanian A R and Kurpad S N (2005). Quiescent neural cells regain multipotent stem cell characteristics influenced by adult neural stem cells in co-culture. *Experimental Neurology* **191**: 193-197.

Alhadlaq A and Mao J J (2003). Tissue-engineered neogenesis of human-shaped mandibular condyle from rat mesenchymal stem cells. *Journal of Dental Research* **82**: 951-956.

Alhadlaq A and Mao J J (2004). Mesenchymal stem cells: Isolation and therapeutics. *Stem Cells and Development* **13**: 436-448.

Alms M (1961). Fracture Mechanics. *The Journal of Bone and Joint Surgery* **43B**(1): 162-166.

Alt E U, Senst C, Murthy S N, Slakey D P, Dupin C L, Chaffin A E, Kadowitz P J and Izapanah R (2012). Aging alters tissue resident mesenchymal stem cell properties. *Stem Cell Research* **8**: 215-225.

Anderson J M, Vines J B, Patterson J L, Chen H, Javed A and Jun H-W (2011). Osteogenic differentiation of human mesenchymal stem cells synergistically enhanced by biomimetic peptide amphiles combined with conditioned medium. *Acta Biomaterialia* **7**: 675-682.

Anuja G I, Latha P G, Suja S R, Shyamal S, Shine V J, Sini S, Pradeep S, Shikha P and Rajasekharan S (2010). Anti-inflammatory and analgesic properties of *D.quercifolia* (L.) J. Smith. *Journal of Ethnopharmacology* **132**: 456-460.

Arora M, Burns L J, Davies S M, MacMillan M L, Defor T E, Miller W J and Weisdorf D J (2003). Chronic graft-versus-host disease: A prospective cohort study. *Biology of Blood and Marrow Transplantation* **9**: 38-45.

Awang D V C (2009). Tyler's Herbs of choice. The therapeutics use of phytomedicinals. 3rd Edition. New York, CRC Press.

Aziz Z and Tey N P (2009). Herbal medicine: Prevalence and predictors of use among Malaysian adults. *Complementary Therapies in Medicine* **17**: 44-50.

Balk M L, Bray J, Day C, Epperly M, Greenberger J, Evans C H and Niyibizi C (1997). Effect of rhBMP-2 on the osteogenic potential of bone marrow stromal cells from an osteogenesis imperfecta mouse (*oim*). *Bone* **21**(1): 7-15.

Banwart J C, Asher M A and Hassanein R S (1995). Iliac crest bone graft harvest donor site morbidity. a statistical evaluation. *Spine* **20**: 1055-1060.

Basha B, Rao D S, Han Z-H and Parfitt A M (2000). Osteomalacia due to Vitamin D Depletion: A Neglected Consequence of Intestinal Malabsorption. *The American Journal of Medicine* **108**: 296-300.

Benetos I S, Babis G C, Zoubos A B, Benetou V and Soucacos P N (2007). Factors affecting the risk of hip fractures. *Injury, International Journal of Care of The Injured* **38**: 735-744.

Berg K M, Kunins H V, Jackson J L, Nahvi S, Chaudhry A, Harris K A J, Malik R and Arnsten J H (2008). Association Between Alcohol Consumption and Both Osteoporotic Fracture and Bone Density. *The American Journal of Medicine* **121**(5): 406-418.

Bosnakovski D, Mizuno M, Kim G, Ishiguro T, Okumura M, Iwanaga T, Kadosawa T and Fujinaga T (2004). Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet cultural system. *Experimental Hematology* **32**: 502-509.

Bostrom M P, Yang X, Kennan M, Sandhu H, Dicarlo E and Lane J M (2001). An unexpected outcome during testing of commercially available demineralized bone graft materials: How safe are the non-allograft components? *Spine* **26**: 1425-1428.

Boyce S T and Warden G D (2002). Principles and practices for treatment of cutaneous wounds with cultured skin substitutes. *American Journal of Surgery* **183**(4): 445-456.

Branski L K, Gauglitz G G, Herndon D N and Jeschke M G (2009). A review of gene and stem cell therapy in cutaneous wound healing. *Burns* **35**(2): 171-180.

Brown K L B and Cruess R L (1982). Bone and cartilage transplantation surgery. *The Journal of Bone and Joint Surgery American* **64-A**: 270-279.

Bruder S P and Chaplan A I (2000). Bone regeneration through cellular engineering. *Principles of Tissue Engineering (Second Edition)*. **Chapter 48:** 683-696.

Bruder S P and Jaiswal N (1995). Transient exposure of human mesenchymal stem cells to dexamethasone is capable of inducing sustained osteogenic lineage progression. *Journal of Bone and Mineral Research* **10**(S41).

Cai Y, Luo Q, Sun M and Corke H (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Lfe Sciences* **74**(17): 2157-2184.

Calixto J B (2000). Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). *Brazillian Journal of Medical and Biological Research* **33**: 179-189.

Canel C, Moraes R M, Dayan F E and Ferreira D (2000). Podophyllotoxin. *Phytochemistry* **54**(2): 115-120.

Chai Y, Abas W W and Kim K (2006). Osteogenic Expression of Bone Marrow Stromal Cells on PCLTF Scaffold: *In vitro* Study. *Asian Journal of Cell Biology* **1**(1): 40-47.

Chang J-K, Li C-J, Liao H-J, Wang C-K, Wang G-J and Ho M-L (2009). Anti-inflammatory drugs suppress proliferation and induce apoptosis through altering expressions of cell cycle regulators and pro-apoptic factors in cultured human osteoblasts. *Toxicology* **258**: 148-156.

Chaplan A I (1991). Mesenchymal stem cells. *Journal of Orthopaedic Research* **9**: 641-650.

Cheung F (2011). TCM: Made in China. *Nature* **480**: S82-S83.

Cheung P-L (2012). Local medicinal herbs are ready to embark. *Sin Chew Daily*. Petaling Jaya.

Chu I, Bodnar J A, White E L and Bowman R N (1996). Quantification of vincristine and vinblastine in *Catharanthus roseus* plants by capillary zone electrophoresis. *Journal of Chromatography A* **755**: 281-288.

Clarke B (2008). Normal Bone Anatomy and Physiology. *Clinical Journal of American Society of Nephrology* **3**: S131-S139.

Clavel C and Verfaillie C M (2008). Bone-marrow-derived cells and heart repair. *Current Opinion in Organ Transplantation* **13**: 36-43.

Compston J (2007). Smoking and the Skeleton. *The Journal of Clinical Endocrinology and Metabolism* **92**(2): 428-429.

Croft A P and Przyborski S A (2004). Mesenchymal stem cells from the bone marrow stroma: basic biology and potential for cell therapy. *Current Anaesthesis & Critical Care* **15**: 410-417.

Cummings S R and Melton I I I L J (2002). Epidemiology and outcomes of osteoporotic fractures. *Lancet* **359**: 1761-1767.

Danisovic L, Varga I and Polak S (2012). Growth factors and chondrogenic differentiation of mesenchymal stem cells. *Tissue and Cell* **44**: 69-73.

Dasi L P, Simon H A, Sucosky P and Yoganathan A P (2009). Fluid mechanics of artificial heart valves. *Clinical and Experimental Pharmacology and Physiology* **36**: 225-237.

Datta A and Srivastava P S (1997). Variation in vinblastine production by *Catharanthus roseus* during *in vivo* and *in vitro* differentiation. *Phytochemistry* **46**(1): 135-137.

Davies J E, Karp J M and Baksh D, Eds. (2002). Mesenchymal cell culture: Bone. *Methods of Tissue Engineering*. US, Academic Press.

Dent C E, Richens A, Rowe D J F and Stamp T C B (1970). Osteomalacia with long-term anticonvulsant therapy in epilepsy. *British Medical Journal* **4**: 69-72.

Dhanwal D K (2011). Thyroid disorders and bone mineral metabolism. *Indian Journal of Endocrinology and Metabolism* **15**(Supplement 2): S107-S112.

Diomidis N, Mischler S, More N S and Roy M (2012). Tribo-electrochemical characterization of metallic biomaterials for total joint replacement. *Acta Biomaterials* **8**: 852-859.

Dominici M, Blanc K L, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D and Horwitz E (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**: 315-317.

Donzelli E, Salvade A, Mimo P, Vigano M, Morrone M, Papagna R, Carini F, Zaopo A, Miloso M, Baldoni M and Tredici G (2007). Mesenchymal stem cells cultured on a collagen scaffold: *In vitro* osteogenic differentiation. *Archives of Oral Biology* **52**: 64-73.

Enneking W F, Eady J L and Burchardt H (1980). Autogenous cortical bone grafts in the reconstruction of segmental skeletal defects. . *Journal of Bone and Joint Surgery American* **62-A**(1039-1058).

Eriksen E F, Axelrod D W and Melsen F (1994). Bone Histomorphometry. *Raven Press* New York**:** 1-12.

Esser K B, Semagn K and Wolde-Yohannes L (2003). Medicinal use and social status of the soap berry *endod* (*Phytolacca dodecandra*) in Ethiopia. *Journal of Ethnopharmacology* **85**: 269-277.

Ferron M, Wei J, Yoshizawa T, Ducy P and Karsenty G (2010). An ELISA-based method to quantify osteocalcin carboxylation in mice. . *Biochemical and Biophysical Research Communications* **397**: 691-696.

Finkemeier C G (2002). Bone-grafting and bone graft substitutes. *The Journal of Bone and Joint Surgery* **84-A**(3): 454-464.

Foundation N O (2002). America's Bone Health: The State of Osteoporosis and Low Bone Mass in Our Nation. Washington, DC, National Osteoporosis Foundation.

Friedenstein A J, Chailakhjan R K and Lalykina K S (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell and Tissue Kinetics* **3**: 393-403.

Friedenstein A J, Piatetzky-Shapiro I I and Petrakova K V (1966). Osteogenesis in transplants of bone marrow cells. *Journal of Embryology and Experimental Morphology* **16**(3): 381-390.

Fukuda K (2002). Reprogramming of bone marrow mesenchymal stem cells into cardiomyocytes. *Comptes Rendus Biologies* **325**: 1027-1038.

Gonzalez-Tejero M R, Casares-Porcel M, Sanchez-Rojas C P, Ramiro-Gutierrez J M, Molero-Mesa J, Pieroni A, Giusti M E, Censorii E, Pasquale C d, Della A, Paraskeva-Hadijchambi D, Hadjichambis A, Houmani Z, El-Demerdash M, El-Zayat M, Hmamouchi M and Eljohrig S (2008). Medicinal plants in the Mediterranean area: Synthesis of the results of the project Rubia. *Journal of Ethnopharmacology* **116**: 341-357.

Gregory C A, Gunn W G, peister A and Prockop D J (2004). An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetypyridinium chloride extraction. *Analytical Biochemistry* **329**: 77-84.

Grigoradis A E, Heersche J N M and Aubin J E (1988). Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone derived clonal cell population: Effect of dexamethasone. *The Journal of Cell Biology* **106**(2139-2151).

Grosvenor P W, Gothard P K, Willam N C M, Supriono A and Gray D O (1995). Medicinal plants from Riau Province, Sumatra, Indonesia. Part 1: Uses. *Journal of Ethnopharmacology* **45**: 75-95.

Grosvenor P W, Supriono A and Gray D O (1995). Medicinal plants from Riau Province, Sumatra, Indonesia. Part 2: antibacterial and antifungal activity. *Journal of Ethnopharmacology* **45**: 97-111.

Gulley M L, Swinnen L J, Plaisance J K T, Shnell C, Grogan T M and Chneider B G (2003). Tumor origin and CD20 expression in posttransplant lymphoproliferative disorder occurring in solid organ transplant recipients: Implications for immune-based therapy. *Transplantation* **76**: 959-964.

Hanada K, Dennis J E and Chaplan A I (1997). Stimulatory effects of basic fibroblast growth factor and bone morphogenetic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. . *Journal of Bone and Mineral Research* **12**(10): 1606-1614.

Haynesworth S E, Baber M and Chaplan A I (1992). Cell surface antigens on human marrow-derived mesenchymal cells are deteced by monoclonal antibodies. *Bone* **13**: 69-80.

Haynesworth S E, Goshima J, Goldberg V M and Chaplan A I (1991). Characterization of cells with osteogenic potential from human marrow. *Bone* **13**(1): 81-88.

Heary R F, Schlenk R P, Sacchieri T A, Barone D and Brotea C (2002). Persistent iliac crest donor site pain: independent outcome assessment. *Neurosurgery* **50**(3): 510-517.

Hensel A, Maas M, Sendker J, Lechtenberg M, Petereit F, Schmidt A D T and Stark T (2011). *Eupatorium perfoliatum* L.: Phytochemistry, traditional use and current applications. *Journal of Ethnopharmacology* **138**: 641-651.

Hildebrandt C, Buth H, Cho S, Impidjati and Thielecke H (2010). Detection of the osteogenic differentiation of mesenchymal stem cells in 2D and 3 D cultures by electrochemical impedence spectroscopy. *Journal of Biotechnology* **148**: 83-90.

Hoareau L and DaSilva E J (1999). Medicinal plants: a re-emerging health aid. *Electronic Journal of Biotechnology* **2**(2): 1-15.

Hoemann C D, El-Gabalawy H and McKee M D (2009). *In vitro* osteogenesis assays: Influence of the primary cell source on alkaline phosphatase activity and mineralization. *Pathologie Biologie* **57**: 318-323.

Holroyd C, Cooper C and Dennison E (2008). Epidemiology of osteoporosis. *Best Practice & Research Clinical Endocrinology & Metabolism* **22**(5): 671-685.

Hong D, Chen H-X, Xue Y, Li D-M, Wan X-C, Ge R and Li J-C (2009). Osteoblastogenic effects of dexamethasone through upregulation of TAZ expression in rat mesenchymal stem cell. *Journal of Steroid Biochemistry and Molecular Biology* **116**: 86-92.

Hughes S P F and Porter R W (1997). Textbook of orthopaedics and fractures. London, Arnold.

Ikeda K, Tsukui T, Kuniko H, Inoue S (2011). Conditional expression of constitutively active estrogen receptor a in osteoblasts increases bone mineral density in mice. *FEBS Letters* **585**: 1303-1309.

Itharat A, Houghton P J, Eno-Amooquaye E, Burke P J, Sampson J H and Raman A (2004). *In vitro* cytotoxic activity of Thai medicinal plants used traditionally to treat cancer. *Journal of Ethnopharmacology* **90**(1): 33-38.

Iu M-F, Kaji H, Sowa H, Naito J, Sugimoto T and Chihara K (2005). Dexamethasone suppresses Smad3 pathway in osteoblastic cells. *Journal of Endocrinology* **185**: 131-138.

Jaiswal N and Bruder S P (1996). The pleiotropic effects of dexamethasone on osteoblast differentiation depend on the developmental state of the responding cells. *Journal of Bone and Mineral Research* **11**: S259.

Jaiswal N, Haynesworth S E, Chaplan A I and Bruder S P (1997). Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*. *Journal of Cellular Biochemistry* **64**(2): 295-312.

Jeong J C, Kang S K, Youn C H, Jeong C W, Kim H M, Lee Y C, Chang Y C and Kim C H (2003). Inhibition of Drynariae Rhizoma extracts on bone resorption mediated by processing of cathepsin K in cultured mouse osteoclasts. *International Immunopharmacology* **3**(12): 1685-1697.

Jeong J C, Lee J W, Yoon C H, Lee Y C, Chung K H, Kim M G and Kim C H (2005). Stimulative effects of Drynariae Rhizoma extracts on the proliferation and differentiation of osteoblastic MC3T3-E1 Cells. *Journal of Ethnopharmacology* **96**(3): 489-495.

Jin K, Mao X O, Batteur S, Sun Y and Greenberg D A (2003). Induction of neuronal markers in bone marrow cells: differential effects of growth factors and patterns of intracellular expression. *Experimental Neurology* **184**: 78-89.

Jin U-H, Kim D-I, Lee T-K, Lee D-N, Kim J-K, Lee I-S and Kim C-H (2006). Herbal formulation, Yukmi-jihang-tang-Jahage, regulates bone resorption by inhibition of phosphorylation mediated by tyrosine kinase Src and cyclooxygenase expression. *Journal of Ethnopharmacology* **106**: 333-343.

Johnell O (1997). The Socioeconomic Burden of Fractures: Today and in the 21st Century. *The American Journal of Medicine* **103**(2A): 20S-25S.

Karmatschek M, Maier I, Seibel M J, Woitge H W, Ziegler R and Armbruster F P (1997). Improved purification of human bone sialoprotein and development of a homologous radioimmunoassay. *Clinical Chemistry* **43**(11): 2076-2082.

Kim D H, Rhim R, Li L, Martha J, Swaim B H, Banco R J, Jenis L G and Tromanhauser S G (2009). Prospective study of iliac crest bone graft harvest site pain and morbidity. *The Spine Journal* **9**: 886-892.

Kobayashi S, Takahashi H E, Ito A, Saito N, Nawata M, Horiuchi H, Ohta H, Ito A, Iorio R, Yamamoto N and Takaoka K (2003). Trabecular minimodeling in human iliac bone. *Bone* **32**: 163-169.

Komiya A, Watanabe A and Fuse H (2011). Herbal medicine in Japan. *Journal of Men's Health* **8**: S15-S18.

Krajden M, Bishai F, Quan C, Mahony J, Brunton J, Rootman D, Zhao J, Lau W, Snell G, Maurer J, Kesten S and Colby D (1995). Multi-organ donor transmission of hepatitis C virus to five solid organ transplant recipients and lack of transmission to corneal transplant recipients. *Clinical and Diagnostic Virology* **3**: 113-121.

Kumar S, Kumar R and Khan A (2011). Medicinal plant resources: Manifestation and prospects of life-sustaining healthcare system. *Continental Journal of Biological Sciences* **4**(1): 19-29.

Lai H Y, Lim Y Y and Kim K H (2010). *Blechnum Orientale* Linn - a fern with potential as antioxidant, anticancer and antibacterial agent. *BMC Complementary and Alternatives Medicine* **10**: 15.

Lane N E (2006). Epidemiology, etiology, and diagnosis of osteoporosis. *American Journal of Obstetrics and Gynecology* **194**: S3-11.

Langer R and Vacanti J P (1993). Tissue Engineering. *Science* **260**: 920-926.

Lefauveau P and Fardellone P (2004). Extraskeletal risk factors for fractures of proximal femur. *Joint Bone Spine* **71**: 14-17.

Liao T S, Yurgelun M B, Chang S-S, Zhang H-Z, Murakami K, Blaine T A, Parisien M V, Kim W, Winchester R J and Lee F Y-I (2005). Recruitment of osteoclast precursors by stromal cell derived factor-1 (SDF-1) in giant cell tumor of bone. *Journal of Orthopaedic Research* **23**: 230-209.

Lindelof B, Dal H, Wolk K and Malmborg N (2005). Cutaneous squamous cell carcinoma in organ transplant recipients: A study of the Swedish cohort With regard to tumor site. . *Archieves of Dermatology* **141**: 447-451.

Liu Y-K, Uemura T, Nemoto A, Yabe T, Fujii N, Ushida T and Tateishi T (1997). Osteopontin involvement in integrin-mediated cell signaling and regulation of expression of alkaline phosphatase during early differentiation of UMR cells. *FEBS Letters* **420**(1): 112-116.

Lubovsky O, Liebergall M, Mattan Y, Weil Y and Mosheiff R (2005). Early diagnosis of occult hip fractures MRI versus CT scan. *Injury, International Journal of Care of The Injured* **36**: 788-792.

Lyons R A, Delahunty A M, Heaven M, McCabe M, Allen H and Nash P (2000). Incidence of childhood fractures in affluent and deprived areas: population based study. *BMJ* **320**: 149.

Maniatopoulos C (1988). Development and characterization of an *in vitro* system permitting osteogenesis by stromal cells isolated from bone marrow of young adult rat. *Faculty of Dentistry*. Toronto, Canada, University of Toronto. **Doctor of philosophy**.

Marthy S and Richter M (1998). Human immunodeficiency virus activity in rib allografts. *Journal of Oral and Maxillofacial Surgery* **56**: 474-476.

Martins A, Duarte A R C, Faria S, Marques A P, Reis R L and Neves N M (2010). Osteogenic induction of hBMSCs by electrospun scaffolds with dexamethasone release functionality. *Biomaterials* **31**: 5875-5885.

Marty and Alan T (1999). The Complete German Commission E Monographs: Therapeutic Guide to HErbal Medicines. *JAMA* **281**(19): 1852-1853.

Merlotti D, Gennari L, Dotta F, Lauro D and Nuti R (2010). Mechanisms of impaired bone strength in type1 and 2 diabetes
Nutrition, Metabolism & Cardiovascular Diseases **20**: 683-690.

Mitruka S N, Griffith B P, Kormos R L, Hattler B G, Pigula F A, Shapiro R, Fung J J and Pham S M (1997). Cardiac operations in solid-organ transplant recipients. *The Annals of Thorasic Surgery* **64**(5): 1270-1278.

Mosmann T R (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**: 65.

Ng T B, Liu F and Wang H X (2004). The antioxidant effects of aqueous and organic extracts of *Panax quinquefolium*, *Panax notoginseng*, *Codonopsis pilosula*, *Pseudostellaria heterophylla* and *Glehnia littoralis*. *Journal of Ethnopharmacology* **93**: 285-288.

Ogston N, Harrison A J, Cheung H F J, Ashton B A and Hampson G (2002). Dexamethasone and retinoic acid differentially regulate growth and differentiation in an immortalised human clonal bone marrow stromal cell line with osteoblastic characteristic. *Steroids* **67**: 895-906.

Oliveira J M, Sousa R A, Kotobuki N, Tadokoro M, Hirose M, Mano J F, Reis R L and Ohgushi H (2009). The osteogenic differentiation of rat bone marrow stromal cells cultured with dexamethasone-loaded carboxymethylchitosan/poly(aminoamine) dendrimer nanoparticles. *Biomaterials* **30**: 804-813.

Olszewski W L, Moscicka M, Zolich D and Machowski Z (2005). Human keratinocyte stem cells survive for months in sodium chloride and can be successfully transplanted. *Transplantation Proceedings* **37**(1): 525-526.

Olszynski W P, Davison K S, Adachi J D, Brown J I, Cummings S R, Hanley D A, Harris S T, Hodsman A B, Kendler D, McClung M R, Miller P D and Yuen C K (2004). Osteoporosis in Men: Epidemiology, Diagnosis, Prevention, and Treatment. *Clinical Therapeutics* **26**(1): 15-28.

Ong S Y, Dai H and Leong K W (2006). Inducing hepatic differentiation of human mesenchymal stem cells in pellet culture. *Biomaterials* **27**: 4087-4097.

Oshina H, Sotome S, Yoshii T, Torigoe I, Sugata Y, Maehara H, Marukawa E, Omura K and Shinomiya K (2007). Effects of continuous dexamethasone treatment on differentiation capabilities of bone marrow-derived mesenchymal cells. *Bone* **41**: 575-583.

Ott H C, Davis B H and Taylor D A (2005). Cell Therapy for Heart Failure - Muscle, Bone Marrow, Blood, and Cardiac-Derived Stem Cells. *Seminars in Thorasic and Cardiovascular Surgery* **17**(4): 348-360.

Parker E, Shiga A and Davies J E, Eds. (2000). Growing human bone *in vitro*. *Bone Engineering*. Toronto, Em Squared Inc.

Peel N F A, Moore D J, Barrington N A, Bax D E and Eastell R (1995). Risk of vertebral fracture and relationship to bone mineral density in steroid treated rheumatoid arthritis. *Annals of the Rheumatic Diseases* **54**: 801-806.

Peng-Zhang, Dai K-r, Yan S-g, Yan W-q, Chao-Zhang, Chen D-q, Bo-Xu and Xu Z-w (2009). Effects of naringin on the proliferation and osteogenic differentiation of human bone mesenchymal stem cell. *European Journal of Pharmacology* **607**: 1-5.

Peterson B E, Bowen W C, Patrene K D, Mars W M, Sullivan A K, Murase N, Boggs S S, Greenberger J S and Goff J P (1999). Bone Marrow as a potential source of hepatic oval cells. *Science* **284**: 1168-1170.

Pittenger M F, Mackay A M, Beck S C, Jaiswal R K, Douglas R, Mosca J D, Moorman M A, Simonetti D W, Craig S and Marshak D R (1999). Multileage potential of adult human mesenchymal stem cells. *Science* **284**: 143-147.

Pittenger M F and Martin B J (2004). Mesenchymal stem cell and their potential as cardiac therapeutics. *Journal of The American Heart Association* **95**: 9-20.

Poliard A, Nifuji A, Lamblin D, Plee E, Forest C and Kellermann O (1995). Controlled conversion of an immortalized mesodermal progenitor cell towards the osteogenic, chondrogenic, or adipogenic pathways. *The Journal of Cell Biology* **130**: 1461-1472.

Polisetti N, Chaitanya V G, Babu P P and Vemuganti G K (2010). Isolation, characterization and differentiation potential of rat bone marrow stromal cells. . *Neurology India* **58**: 201-208.

Poonam S, Krishna R, Manju B and Vibha T (2005). Preliminary Studies on Activity of *Ocimum sanctum*, *D.quercifolia*, and *Annona squamosa* Against *Neisseria gonorrhoeae*. *Journal of the American Sexually Transmitted Disease Association* **32**(2): 106-111.

Poulsom R, Alison M R, Cook T, Jeffery R, Ryan E, Forbes S J, Hunt T, Wyles S and Wright N A (2003). Bone marrow stem cells contribute to healing of the kidney. *Journal of American Society of Nephrology* **14**: S48-S54.

Price F D, Kuroda K and Rudnicki M A (2007). Stem cell based therapies to treat muscular dystrophy. *Biochemica et Biophysica Acta* **1772**(2): 272-283.

Qu Q, Perala-Heape M, Kapanen A, Dahllund J, Salo J, Vaananen H K and Harkonen P (1998). Estrogen enhances differentiation of osteoblasts in mouse bone marrow culture. *Bone* **22**(3): 201-209.

Quasnichka H L, Tarlton J F, Anderson-MacKenzie J M, Billingham M E J, Bailey A J and Pickford A R (2005). Development of an assay for the quantification of type 1 collagen synthesis in the guinea pig. *Journal of Immunological Methods* **297**: 133-141.

Ramesh N, Viswanathan M B, Saraswathy A, Balakrishna K, Brindha P and Lakshmanaperumalsamy P (2001). Phytochemical and antimicrobial studies on *D.quercifolia*. *Fitoterapia* **72**(8): 934-936.

Renault V, Thornell L-E, Butler-Browne G and Mouly V (2002). Human skeletal muscle satellite cells: aging, oxidative stress and the mitotic clock. *Experimental Gerontology* **37**(10-11): 1229-1236.

Riis B J, Hansen M A, Jensen A M, Overgaard K and Christiansen C (1996). Low bone mass and fast rate of bone loss at menopause: equal risk factors for future fracture: A 15-year
follow-up study. *Bone* **19**(1): 9-12.

Rizzoli R (2008). Nutrition: its role in bone health. *Best Practice & Research Clinical Endocrinology & Metabolism* **22**(5): 813-829.

Rocha A B d, Lopes R M and Schwartsmann G (2001). Natural products in anticancer therapy. *Current Opinion in Pharmacology* **1**(4): 364-369.

Romas E (2008). Corticosteroid-induced osteoporosis and fractures. *Australian Prescriber* **31**(2): 45-49.

Roodman G D (1999). Cell biology of the osteoclast. *Experimental Hematology* **27**: 1229-1241.

Roostaeian J, Carlsen B, Simhaee D, Jarrahy R, Huang W, Ishida K, Rudkin G H, Yamaguchi D T and miller T A (2006). Characterization of growth and osteogenic differentiation of rabbit bone marrow stromal cells. *Journal of Surgical Research* **133**: 76-83.

Ross M H, Romrell L J and Kaye G I (1995). Histology: A text and atlas. 3rd edition. . USA.

Sagar J, Chaib B, Sales K, Winslet M and Seifalian A (2007). Role of stem cells in cancer therapy and cancer stem cells: a review. *Cancer Cell International* **7**: 1-11.

Saleem T K M, Azeem A K, Dilip C, Sankar C, Prasanth N V and Duraisami R (2011). Anti-inflammatory activity of the leaf extracts of *Gendarussa vulgaris* Nees. *Asian Pacific Journal of Tropical Biomedicine* **1**: 147-149.

Schwartz C E, Martha J F, Kowalski P, Wang D A, Bode R, Li L and Kim D H (2009). Prospective evaluation of chronic pain associated with posterior autologous iliac crest bone graft harvest and its effect on postoperative outcome. *Health and Quality of Life Outcomes* **7**(1): 49.

Seshi B, Kumar S and Sellers D (2000). Human bone marrow stromal cell: Coexpression of markers specific for multiple mesenchymal cell lineages. *Blood Cells, Molecules, and Diseases* **26**(3): 234-2346.

Shah K (2012). Mesenchymal stem cells engineered for cancer therapy. *Advanced Drug Delivery Reviews* **64**: 739-748.

Silber J S, Anderson D G, Daffner S D, Brislin B T, Leland J M, Hilibrand A S, Vaccaro A R and Albert T J (2003). Donor site morbidity after anterior iliac crest bone harvest for single-level anterior cervical discectomy
and fusion. *Spine* **28**(2): 134-139.

Sipe J D (2002). Tissue engineering and reparative medicine. *Annals New York Academy of Sciences* **961**: 1-9.

Smit H F, Woerdenbag H J, Singh R H, Meulenbeld G J, Labadie R P and Zwaving J H (1995). Ayurvedic herbal drugs with possible cytostatic activity. *Journal of Ethnopharmacology* **47**(2): 75-84.

Spees J L, Whitney M J, Sullivan D E, Lasky J A, Laboy M, Ylostalo J and Prockop D J (2007). Bone marrow progenitor cells contribute to repair and remodeling of the lung and heart in a rat model of progressive pulmonary hypertension. *The FASED Journal* **22**(4): 1226-236.

Stolzing A and Scutt A (2006). Age-related impairment of mesenchymal progenitor cell function. *Aging Cell* **5**(213-224).

Strohl W R (2000). The role of natural products in a modern drug discovery program. *Drug Discovery Today* **5**(2): 39-41.

Sun J S, Lin C Y, Dong G C, Sheu S Y, Lin F H, Chen L T and Wang Y J (2002). The effect of Gu-Sui-Bu (*Drynaria fortunei* J. Sm) on bone cell activities. *Biomaterials* **23**(16): 3377-3385.

Taichman R S (2005). Blood and bone: Two tissues whose fates are intertwined to create the hematopoietic stem cell niche. *Blood* **105**: 2631-2639.

Terkeltaub R A (2001). Inorganic pyrophosphaste generation and disposition in pathophysiology. *Americans Journal of Physiology - Cell Physiology* **281**: C1-C11.

Thomson J A, Itskovitz-Eldor J, Shapiro S S, Waknitz M A, Swiergiel J J, Marshall V S and Jones J M (1998). Embryonic stem cell lines derived from human blastocysts. *Science* **282**: 1145-1147.

Treasure J. 5.1 Introduction to side effects, safety and toxicity of medicinal herbs. *Henriette's Herbal Homepage*, 2011, from http://www.henriettesherbal.com/faqs/medi-5-1-side-effects.html.

Trentz O A, Arikketh D, Sentilnathan V, Hemmi S, Handschin A E, Rosario B d, Mohandas P and Mohandas P V A (2010). Surface proteins and osteoblast markers: characterization of human adipose tissue-derived osteogenic cells. *European Journal of Trauma and Emergency Surgery* **36**: 457-463.

Tung H (2010). Acupuncture + Bo-Gu-Cao (*Gendarussa vulgaris*) heals bone fracture. *Healthy Life*. Ipoh, Perak, Malaysia, Munsang Poh (M) Sdn. Bhd. **58:** 10-11.

Urasopon N, Hamada Y, Asaoka K, Cherdshewasart W and Malaivijitnond S (2007). *Pueraria mirifica*, a phytoestrogen-rich herb, prevents bone loss in orchidectomized rats. *Maturitas* **56**(3): 322-331.

Vater C, Kasten P and Stiehler M (2011). Culture media for the differentiation of mesenchymal stromal cells. *Acta Biomaterialia* **7**: 463-477.

Vija L, Farge D, Gautier J F, Vexiau P, Dumitrache C, Bourgarit A, Verrecchia F and Larghero J (2009). Mesenchymal stem cells: Stem cel therapy perspectives for type 1 diabetes. *Diabetes & Metabolism* **35**(2): 85-93.

Wakdikar S and Marg K (2004). Global health care challenge: Indian experiences and new prescription. *Biotechnology Issues for Developing Countries* **7**(3): 1-5.

Walsh S, Jordan G R, Jefferiss C, Stewart K and Beresford J N (2001). High concentrations of dexamethasone suppress the proliferation but not the differentiation or further maturation of human osteoblast precursors *in vitro*: relevance to glucocorticoid-induced osteoporosis. *Rheumatology* **40**: 74-83.

Wang S, Zheng Z, Weng Y, Yu Y, Zhang D, Fan W, Dai R, Hu Z (2004). Angiogenesis and antiangiogenensis activity of Chinese medicinal herbal extracts. *Life Sciences* **74**: 2467-2478.

Wang X, Zhen L, Zhang G, Wong M, Qin L, Yao X (2011). Osteogenic effects of flavonoid aglycones from an osteoprotective fraction of *Drynaria fortunei* - An *in vitro* efficacy study. *Phytomedicine* **18**: 868-872.

Wang X, Wu J, Chiba H, Yamada K and Ishimi Y (2005). *Puerariae radix* prevents bone loss in castrated male mice *Metabolism* **54**(11): 1536-1541.

White S C, Atchison K A, Gornbein J A, Nattiv A, Paganini-Hill A and Service S K (2006). Risk Factors for Fractures in Older Men and Women: The Leisure World Cohort Study. *Gender Medicine* **3**(2): 110-123.

Yoo KM, Choong HL, Lee H, Moon BK, Lee CY (2008). Relative antioxidant and cytoprotective activities of common herbs. *Food Chemistry* **106**: 929-936.

Zhang W, Ma D, Zhao Q and Ishida T (2010). The effect of the major components of *Fructus cnidii* on osteoblasts *in vitro*. *Journal of Acupuncture and Meridian Studies* **3**(1): 32-37.

APPENDICES

Appendix 1: Ethical approval from Animal Ethics Committee, Faculty of Medicine, University of Malaya

→ Poon Chi Tet

UNIVERSITI MALAYA
KUALA LUMPUR

يونيورسيتي مالايا

SETIAUSAHA

16 April 2008

Professor Dr Ir Wan Abu Bakar Wan Abas
Department Biomedical Engineering
Faculty of Engineering
Universiti Malaya

Tuan,

HERBS IN ENCHANCING OSTEOBLAST CELL GROWTH IN SCAFFOLD

Dengan sukacitanya Jawatankuasa Etika Penjagaan dan Penggunaan Haiwan, Fakulti Perubatan, Universiti Malaya telah meluluskan permohonan untuk penyelidikan tersebut di atas.

No rujukan etika: **BE/16/04/2008/ WAB (R)**

Sila ambil perhatian bahawa nombor rujukan etika yang diberi adalah sah untuk tempoh masa dua tahun sehingga 15 April 2010. Selepas menamatkan penyelidikan tersebut, untuk mendapatkan perkhidmatan *Euthanasia* dan pembakaran haiwan, sila menghubungi Puan Hamidah Hamid, talian telefon 03-7967 4798/ 4792.

Sekian, terima kasih.

Yang benar,

Dr. Haji Azizuddin Bin Haji Kamaruddin
Ketua
Pusat Haiwan Makmal
Fakulti Perubatan
Merangkap Setiausaha Jawatankuasa Etika Penjagaan dan Penggunaan Haiwan

SK : **Cik Zura Hazleena Hamizan**
Setiausaha MCRC
Pejabat Dekan
Fakulti Perubatan

Jawatankuasa Penjagaan Dan Penggunaan Haiwan,
Pusat Haiwan Makmal, Fakulti Perubatan, Universiti Malaya,
50603 Kuala Lumpur, Malaysia
Tel : (603) 7967 4795 Fax : (603) 7955 9886 Email : azizud@um.edu.my

Appendix 2: Raw data of generating standard curve of CCK-8 assay.

OPTIMA
BMG LABTECH

Testname: CCK-8 2010/11/16 15:15:23
ID 1,2,3: CCK-8 standard 17/11/10 662.dbf

Hide protocol settings

Absorbance, end point
Microplate: TPP 96

Kinetic window	1	2	3	4
No. of cycles	1	-	-	-
Meas. start time [s]	0.0	-	-	-
No. of flashes	20	-	-	-

No.	Excitation	Emission
1	Em460	empty

Pos. delay [s]: 0.5
Reading direct. 9

Calculation Start1: 1 Stop1: 1 Start2: 0 Stop2: 0

Comment:

Calculation: Sum Range1 1 conter Raw data

Table 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.671	0.666	0.667	0.677	0.659	0.640	0.629	0.616	0.655	0.658	0.649	0.659
B	0.784	1.153	1.476	1.466	1.689	2.025	2.025	2.098	2.158	2.297	2.429	2.517
C	0.846	0.924	1.439	1.641	1.870	1.656	1.880	1.959	2.351	2.572	2.368	2.312
D	0.853	0.941	1.278	1.446	1.615	1.883	1.756	1.826	1.953	2.315	2.498	2.608
E	0.810	1.145	1.175	1.176	1.393	1.724	1.685	2.148	1.914	2.257	2.339	2.401
F	0.785	0.875	1.354	1.589	1.692	1.456	1.764	1.589	1.809	2.092	2.358	2.465
G	0.790	1.044	1.331	1.363	1.465	1.549	1.635	1.565	1.791	1.982	2.334	2.468
H	0.653	0.663	0.635	0.618	0.639	0.623	0.585	0.610	0.595	0.620	0.626	0.644

Appendix 3: Raw data of proliferation rate of rBMS derived osteoblasts after incubation with herbal extracts, (a) 3 days, (b) 7 days and (c) 14 days.

(a)

OPTIMA
BMG LABTECH

Testname: CCK-8 2011/08/29 14:46:24
ID 1,2,3: Herbal Extract 3 days 1 1084.dbf

Hide protocol settings

Absorbance, end point
Microplate: TPP 96

No.	Excitation	Emission
1	Em460	empty

Pos. delay [s]: 0.5
Reading direct. 9

Kinetic window	1	2	3	4
No. of cycles	1	-	-	-
Meas. start time [s]	0.0	-	-	-
No. of flashes	20	-	-	-

Calculation Start1: 1 Stop1: 1 Start2: 0 Stop2: 0

Comment:

Calculation: Sum Range1 1 conter Raw data

Table 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.734	0.673	0.685	0.674	0.658	0.655	0.646	0.638	0.650	0.655	0.661	0.663
B	1.153	1.223	1.173	1.082	1.076	1.111	1.079	1.047	1.292	1.293	1.214	1.238
C	1.157	1.254	1.309	1.289	1.367	1.266	1.265	1.172	1.220	1.248	1.233	1.219
D	1.115	1.248	1.262	1.262	1.458	1.433	1.372	1.295	1.243	1.254	1.300	1.243
E	1.094	1.146	1.211	1.275	1.410	1.409	1.458	1.254	1.245	1.208	1.241	1.283
F	1.062	1.118	1.190	1.284	1.387	1.440	1.451	1.233	1.166	1.234	1.221	1.245
G	1.159	1.201	1.168	1.130	1.204	1.246	1.244	1.060	1.155	1.237	1.283	1.271
H	0.706	1.191	1.225	1.196	1.211	1.175	1.230	0.031	0.032	0.032	0.032	0.031

(b)

OPTIMA
BMG LABTECH

Testname: CCK-8 2011/09/02 16:00:56
ID 1,2,3: Herbal Extract 7 days 1 1109.dbf

Absorbance, end point
Microplate: TPP 96

Kinetic window	1	2	3	4
No. of cycles	1	-	-	-
Meas. start time [s]	0.0	-	-	-
No. of flashes	20	-	-	-

No.	Excitation	Emission
1	Em460	empty

Pos. delay [s]: 0.5
Reading direct. 9

Calculation Start1: 1 Stop1: 1 Start2: 0 Stop2: 0

Comment:

Calculation: Sum Range1 1 Raw data

Table 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.505	0.495	0.497	0.492	0.497	0.489	0.481	0.485	0.498	0.488	0.496	0.500
B	1.106	1.662	1.403	1.402	1.757	1.725	1.822	1.689	1.718	1.831	1.815	1.580
C	1.117	1.729	1.409	1.502	1.819	1.815	1.798	1.776	1.596	1.657	1.585	1.844
D	1.084	1.623	1.372	1.470	1.878	1.881	1.881	1.809	1.689	1.873	1.865	1.867
E	1.159	1.633	1.327	1.366	1.801	1.911	1.974	1.690	1.747	1.849	1.605	1.846
F	1.112	1.662	1.300	1.350	1.800	1.894	2.081	1.832	1.754	1.905	1.628	1.505
G	1.156	1.591	1.483	1.484	1.867	1.859	1.959	1.855	1.660	1.811	1.767	1.639
H	0.539	1.435	1.252	1.212	1.208	1.281	1.299	0.036	0.035	0.034	0.032	0.035

(c)

OPTIMA
BMG LABTECH

Testname: CCK-8 2011/09/09 15:13:57
ID 1,2,3: Herbal Extract 14 days 1 1142.dbf

Absorbance, end point
Microplate: TPP 96

No.	Excitation	Emission
1	Em460	empty

Pos. delay [s]: 0.5
Reading direct. 9

Kinetic window	1	2	3	4
No. of cycles	1	-	-	-
Meas. start time [s]	0.0	-	-	-
No. of flashes	20	-	-	-

Calculation Start1: 1 Stop1: 1 Start2: 0 Stop2: 0

Comment:

Calculation: Sum Range1 1 conter Raw data

Table 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.411	0.422	0.411	0.403	0.421	0.409	0.405	0.400	0.407	0.426	0.409	0.408
B	1.375	2.125	1.573	1.809	1.667	1.908	1.647	2.069	2.119	2.044	1.899	2.097
C	1.301	2.290	1.709	1.844	1.914	1.720	1.922	2.155	1.957	1.958	1.957	1.925
D	1.237	2.352	1.654	2.108	2.077	1.822	2.110	2.578	1.897	2.257	2.180	2.126
E	1.256	2.326	1.747	1.819	1.921	1.991	2.279	2.170	1.940	2.153	1.842	1.869
F	1.281	2.557	1.570	1.976	1.972	2.107	1.999	2.038	1.907	1.653	1.648	2.196
G	1.437	2.367	1.696	1.850	2.073	1.734	2.006	1.647	1.755	1.576	1.501	2.146
H	0.424	1.890	1.394	1.623	1.677	1.407	1.504	0.390	0.041	0.040	0.044	0.050

Appendix 4: Raw data of 4-nitrophenol standard curve.

User: USER	Path: C:\Program Files\BMG\OPTIMA\User\Data\		Test ID: 234
			Time: 6:23:33
Test Name: ALP		Date: 6/21/2010	PM
ID1: ALP standard			
Absorbance	Absorbance values are displayed as OD		

Raw Data (405-10)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.21	0.227	0.324	0.351	0.391	0.441	0.497	0.488	0.558	0.611	0.628	0.679
B	0.176	0.226	0.3	0.339	0.388	0.465	0.465	0.534	0.541	0.605	0.662	0.841
C	0.2	0.2	0.316	0.342	0.38	0.435	0.466	0.49	0.523	0.574	0.64	0.719
D	0.175	0.197	0.334	0.357	0.385	0.431	0.506	0.576	0.61	0.645	0.622	0.819
E	0.211	0.209	0.322	0.339	0.417	0.438	0.502	0.545	0.615	0.656	0.634	0.654
F	0.185	0.194	0.303	0.36	0.397	0.442	0.47	0.549	0.584	0.575	0.649	0.616
G	0.193	0.194	0.32	0.35	0.395	0.44	0.465	0.531	0.571	0.567	0.622	0.596
H	0.028	0.03	0.029	0.029	0.027	0.028	0.029	0.031	0.029	0.03	0.03	0.03

Appendix 5: One-Way ANOVA analysis of proliferation rate of rBMS derived osteoblast after incubation with 12 types of herbal extract for (a) day 3, (b) day 7 and (c) day 14 by software IBM SPSS Statistics.

(a)

Multiple Comparisons

Cell number

Tukey HSD

(I) Type of sample	(J) Type of sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	DQ EtOH	1373.33333	639.07495	.630	-824.3211	3570.9878
	DQ Hex	-330.20833	639.07495	1.000	-2527.8628	1867.4461
	DQ EtAc	-436.25000	639.07495	1.000	-2633.9044	1761.4044
	DQ H2O	-592.91667	639.07495	.999	-2790.5711	1604.7378
	GV EtOH	-2005.62500	639.07495	.108	-4203.2794	192.0294
	GV Hex	-2041.66667	639.07495	.094	-4239.3211	155.9878
	GV EtAC	-2084.37500	639.07495	.079	-4282.0294	113.2794
	GV H2O	-500.62500	639.07495	1.000	-2698.2794	1697.0294
	GVF EtOH	775.62500	639.07495	.991	-1422.0294	2973.2794
	GVF Hex	61.66667	639.07495	1.000	-2135.9878	2259.3211
	GVF EtAC	146.66667	639.07495	1.000	-2050.9878	2344.3211
	GVF H2O	12.70833	639.07495	1.000	-2184.9461	2210.3628

*. The mean difference is significant at the 0.05 level.

(b)

Multiple Comparisons

Cell number

Tukey HSD

(I) Type of sample	(J) Type of sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	DQ EtOH	1569.58333	607.52977	.341	-519.5933	3658.7600
	DQ Hex	-5163.75000*	607.52977	.000	-7252.9267	-3074.5733
	DQ EtAc	-1785.20833	607.52977	.170	-3874.3850	303.9683
	DQ H2O	-2428.33333*	607.52977	.010	-4517.5100	-339.1567
	GV EtOH	-7264.37500*	607.52977	.000	-9353.5517	-5175.1983
	GV Hex	-7706.66667*	607.52977	.000	-9795.8433	-5617.4900
	GV EtAC	-8692.70833*	607.52977	.000	-10781.8850	-6603.5317
	GV H2O	-6846.87500*	607.52977	.000	-8936.0517	-4757.6983
	GVF EtOH	-5667.08333*	607.52977	.000	-7756.2600	-3577.9067
	GVF Hex	-7380.20833*	607.52977	.000	-9469.3850	-5291.0317
	GVF EtAC	-5903.95833*	607.52977	.000	-7993.1350	-3814.7817
	GVF H2O	-5884.58333*	607.52977	.000	-7973.7600	-3795.4067

*. The mean difference is significant at the 0.05 level.

(c)

Multiple Comparisons

Cell number

Tukey HSD

(I) Type of sample	(J) Type of sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	DQ EtOH	3182.08333	1299.16436	.424	-1285.4902	7649.6568
	DQ Hex	-9451.04167*	1299.16436	.000	-13918.6152	-4983.4682
	DQ EtAc	-1116.87500	1299.16436	1.000	-5584.4485	3350.6985
	DQ H2O	-4246.25000	1299.16436	.078	-8713.8235	221.3235
	GV EtOH	-4478.33333*	1299.16436	.049	-8945.9068	-10.7598
	GV Hex	-3917.70833	1299.16436	.143	-8385.2818	549.8652
	GV EtAC	-5387.08333*	1299.16436	.006	-9854.6568	-919.5098
	GV H2O	-6898.95833*	1299.16436	.000	-11366.5318	-2431.3848
	GVF EtOH	-4551.45833*	1299.16436	.042	-9019.0318	-83.8848
	GVF Hex	-4452.70833	1299.16436	.052	-8920.2818	14.8652
	GVF EtAC	-3386.25000	1299.16436	.328	-7853.8235	1081.3235
	GVF H2O	-6176.25000*	1299.16436	.001	-10643.8235	-1708.6765

*. The mean difference is significant at the 0.05 level.

Appendix 6: One-Way ANOVA analysis of ALP detection assay of rBMS derived osteoblast after incubation with 12 types of herbal extract on (a) day 7, (b) day 14 and (c) day 21 by software IBM SPSS Statistics.

(a)

Multiple Comparisons

ALP Activity/Total protein

Tukey HSD

(I) Type of sample	(J) Type of sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	DQ EtOH	-.001781365	.000854667	.674	-.00472040	.00115767
	DQ Hex	-.001021628	.000854667	.992	-.00396066	.00191740
	DQ EtAc	.000140353	.000854667	1.000	-.00279868	.00307938
	DQ H2O	-.003371458*	.000854667	.011	-.00631049	-.00043243
	JG EtOH	.000128436	.000854667	1.000	-.00281060	.00306747
	JG Hex	.003286043*	.000854667	.016	.00034701	.00622507
	JG EtAC	.000095640	.000854667	1.000	-.00284339	.00303467
	JG H2O	-.006902350*	.000854667	.000	-.00984138	-.00396332
	BGC EtOH	.003253785*	.000854667	.017	.00031475	.00619282
	BGC Hex	.004161491*	.000854667	.001	.00122246	.00710052
	BGC EtAC	.003162496*	.000854667	.024	.00022346	.00610153
	BGC H2O	.001519514	.000854667	.853	-.00141952	.00445855

*. The mean difference is significant at the 0.05 level.

(b)

Multiple Comparisons

ALP Activity/Total protein

Tukey HSD

(I) Type of sample	(J) Type of sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	DQ EtOH	.008606625*	.001589714	.000	.00313991	.01407334
	DQ Hex	.009352206*	.001589714	.000	.00388549	.01481892
	DQ EtAc	.012216806*	.001589714	.000	.00675009	.01768352
	DQ H2O	.009398884*	.001589714	.000	.00393217	.01486560
	JG EtOH	.008066511*	.001589714	.000	.00259979	.01353323
	JG Hex	.015520841*	.001589714	.000	.01005412	.02098756
	JG EtAC	.012986829*	.001589714	.000	.00752011	.01845355
	JG H2O	-.003896976	.001589714	.423	-.00936369	.00156974
	BGC EtOH	.010719402*	.001589714	.000	.00525268	.01618612
	BGC Hex	.012195437*	.001589714	.000	.00672872	.01766215
	BGC EtAC	.012216218*	.001589714	.000	.00674950	.01768294
	BGC H2O	.003835658	.001589714	.449	-.00163106	.00930238

*. The mean difference is significant at the 0.05 level.

(c)

Multiple Comparisons

ALP Activity/Total protein

Tukey HSD

(I) Type of sample	(J) Type of sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	DQ EtOH	-.012522591*	.003044838	.007	-.02299320	-.00205199
	DQ Hex	-.007167475	.003044838	.488	-.01763808	.00330313
	DQ EtAc	-.008745130	.003044838	.196	-.01921573	.00172548
	DQ H2O	-.013971985*	.003044838	.001	-.02444259	-.00350138
	JG EtOH	.005480901	.003044838	.842	-.00498970	.01595151
	JG Hex	.011143197*	.003044838	.027	.00067259	.02161380
	JG EtAC	.006908223	.003044838	.547	-.00356238	.01737883
	JG H2O	-.011806090*	.003044838	.014	-.02227670	-.00133548
	BGC EtOH	.009386609	.003044838	.123	-.00108400	.01985721
	BGC Hex	.011459882*	.003044838	.020	.00098928	.02193049
	BGC EtAC	.004056480	.003044838	.980	-.00641413	.01452709
	BGC H2O	-.011498888*	.003044838	.019	-.02196949	-.00102828

*. The mean difference is significant at the 0.05 level.

Appendix 7: One-Way ANOVA analysis of proliferation rate of dose dependency evaluation of *D.quercifolia and J.gendarussa* on rBMS derived osteoblast after incubation for (a) day 3, (b) day 7, (c) day 14 and (d) day 21 by software IBM SPSS Statistics.

(a)

Multiple Comparisons

Cell number

Tukey HSD

(I) Herb concentration	(J) Herb concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	DQ 10	220.41667	213.49436	.993	-496.3998	937.2331
	DQ 50	-278.75000	213.49436	.964	-995.5664	438.0664
	DQ 150	-103.75000	213.49436	1.000	-820.5664	613.0664
	DQ 250	612.29167	213.49436	.161	-104.5248	1329.1081
	DQ 500	1100.62500*	213.49436	.000	383.8086	1817.4414
	GV 10	-181.66667	213.49436	.999	-898.4831	535.1498
	GV 50	-62.29167	213.49436	1.000	-779.1081	654.5248
	GV 150	-342.08333	213.49436	.875	-1058.8998	374.7331
	GV 250	-198.75000	213.49436	.997	-915.5664	518.0664
	GV 500	228.75000	213.49436	.991	-488.0664	945.5664

*. The mean difference is significant at the 0.05 level.

(b)

Multiple Comparisons

Cell number

Tukey HSD

(I) Herb concentration	(J) Herb concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	DQ 10	-2597.29167*	445.81692	.000	-4094.1411	-1100.4423
	DQ 50	-3528.95833*	445.81692	.000	-5025.8077	-2032.1089
	DQ 150	-2428.95833*	445.81692	.000	-3925.8077	-932.1089
	DQ 250	-1180.83333	445.81692	.250	-2677.6827	316.0161
	DQ 500	623.33333	445.81692	.944	-873.5161	2120.1827
	GV 10	-2306.45833*	445.81692	.000	-3803.3077	-809.6089
	GV 50	-1948.54167*	445.81692	.003	-3445.3911	-451.6923
	GV 150	-2032.91667*	445.81692	.001	-3529.7661	-536.0673
	GV 250	-1975.83333*	445.81692	.002	-3472.6827	-478.9839
	GV 500	-1604.37500*	445.81692	.026	-3101.2244	-107.5256

*. The mean difference is significant at the 0.05 level.

(c)

Multiple Comparisons

Cell number

Tukey HSD

(I) Herb concentration	(J) Herb concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	DQ 10	-3493.33333*	466.80352	.000	-5060.6461	-1926.0205
	DQ 50	-4027.70833*	466.80352	.000	-5595.0211	-2460.3955
	DQ 150	-4252.70833*	466.80352	.000	-5820.0211	-2685.3955
	DQ 250	-2566.45833*	466.80352	.000	-4133.7711	-999.1455
	DQ 500	915.20833	466.80352	.676	-652.1045	2482.5211
	GV 10	-3113.12500*	466.80352	.000	-4680.4378	-1545.8122
	GV 50	-2320.20833*	466.80352	.000	-3887.5211	-752.8955
	GV 150	-1797.08333*	466.80352	.013	-3364.3961	-229.7705
	GV 250	-2171.45833*	466.80352	.001	-3738.7711	-604.1455
	GV 500	-1466.87500	466.80352	.086	-3034.1878	100.4378

*. The mean difference is significant at the 0.05 level.

(d)

Multiple Comparisons

Cell number

Tukey HSD

(I) Herb concentration	(J) Herb concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	DQ 10	-2555.20833	1169.65576	.525	-6482.3776	1371.9609
	DQ 50	-5912.29167*	1169.65576	.000	-9839.4609	-1985.1224
	DQ 150	-6369.37500*	1169.65576	.000	-10296.5443	-2442.2057
	DQ 250	-3965.41667*	1169.65576	.046	-7892.5859	-38.2474
	DQ 500	857.70833	1169.65576	1.000	-3069.4609	4784.8776
	GV 10	-2893.33333	1169.65576	.342	-6820.5026	1033.8359
	GV 50	-3103.54167	1169.65576	.248	-7030.7109	823.6276
	GV 150	-2643.12500	1169.65576	.475	-6570.2943	1284.0443
	GV 250	-3090.41667	1169.65576	.253	-7017.5859	836.7526
	GV 500	-260.20833	1169.65576	1.000	-4187.3776	3666.9609

*. The mean difference is significant at the 0.05 level.

Appendix 8: One-Way ANOVA analysis of ALP activity detection of dose dependency evaluation of *D.quercifolia and J.gendarussa* on rBMS derived osteoblast after incubation for (a) day 7, (b) day 14 and (c) day 21 by software IBM SPSS Statistics.

(a)

Multiple Comparisons

ALP activity/Total protein

Tukey HSD

(I) Herb concentration	(J) Herb concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	DQ 10	-.01476	.00906	.863	-.0452	.0157
	DQ 50	-.01233	.00906	.953	-.0428	.0181
	DQ 150	-.00737	.00906	.999	-.0378	.0231
	DQ 250	-.02475	.00906	.213	-.0552	.0057
	DQ 500	-.00489	.00906	1.000	-.0353	.0255
	GV 10	-.00359	.00906	1.000	-.0340	.0268
	GV 50	-.00602	.00906	1.000	-.0364	.0244
	GV 150	-.00869	.00906	.996	-.0391	.0217
	GV 250	-.00879	.00906	.996	-.0392	.0216
	GV 500	.00345	.00906	1.000	-.0270	.0339

*. The mean difference is significant at the 0.05 level.

(b)

Multiple Comparisons

ALP activity/Total protein

Tukey HSD

(I) Herb concentration	(J) Herb concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	DQ 10	-.01333	.01026	.965	-.0478	.0211
	DQ 50	-.02863	.01026	.189	-.0631	.0058
	DQ 150	-.02547	.01026	.337	-.0599	.0090
	DQ 250	.00763	.01026	1.000	-.0268	.0421
	DQ 500	-.00190	.01026	1.000	-.0363	.0325
	GV 10	-.00663	.01026	1.000	-.0411	.0278
	GV 50	-.00644	.01026	1.000	-.0409	.0280
	GV 150	-.07642*	.01026	.000	-.1109	-.0420
	GV 250	-.07605*	.01026	.000	-.1105	-.0416
	GV 500	-.04411*	.01026	.003	-.0786	-.0097

*. The mean difference is significant at the 0.05 level.

(c)

Multiple Comparisons

ALP activity/Total protein

Tukey HSD

(I) Herb concentration	(J) Herb concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	DQ 10	-.02719	.01508	.773	-.0778	.0234
	DQ 50	-.05563*	.01508	.020	-.1063	-.0050
	DQ 150	-.04757	.01508	.083	-.0982	.0031
	DQ 250	.00480	.01508	1.000	-.0458	.0554
	DQ 500	-.02258	.01508	.914	-.0732	.0280
	GV 10	-.01736	.01508	.985	-.0680	.0333
	GV 50	-.01080	.01508	1.000	-.0614	.0398
	GV 150	-.05845*	.01508	.012	-.1091	-.0078
	GV 250	-.06296*	.01508	.005	-.1136	-.0123
	GV 500	-.05842*	.01508	.012	-.1090	-.0078

*. The mean difference is significant at the 0.05 level.

SELECTED PUBLICATIONS

1. C.T. Poon, W.A.B. Abas, K.H. Kim and B. Pingguan-Murphy (2011). Effect of Herbal Extracts on Rat Bone Marrow Stromal Cells (BMSCs) Derived Osteoblast-Preliminary Result. 5th Kuala Lumpur International Conference on Biomedical Engineering 2011. Vol 35.819-822

Effect of Herbal Extracts on Rat Bone marrow Stromal Cells (BMSCs) Derived Osteoblast – Preliminary Result

C.T. Poon[1], W.A.B. Abas[1], K.H. Kim[2], B.P. Murphy[1]

[1] Department of Biomedical Engineering, Faculty of Engineering, Kuala Lumpur, Malaysia
[2] Department of Physiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Abstract— **Local medicinal herbs are widely used in the management of bone fracture and osteoporosis. However there is no scientific evidence for the significance of the usage of the herbs in bone-related disease treatment. Therefore we initiated proper scientific studies to test the effects of two herbs (*Drynaria quercifolia, Gendarussa vulgaris*) and a formulation powder containing *G.vulgaris*. Here we report on the effect of the extracts of the herbs on the proliferation of rat BMSC derived osteoblasts. Samples of the herbs were obtained from local herbalists and prepared to obtain four types of polarity compound solvent extract for each herb were reconstituted in media tested on rat-BMSC-derived osteoblasts. Rat-BMSC-derived osteoblasts were harvested and seeded in 96-well plates at a density of 3000cells/well. Cells in the wells were treated with 100µg/ml reconstituted extracts and cultured for 3 days, 7 days and 10 days. The resulting cell viability was determined with a proliferation assay using Cell Counting Kit-8 (Sigma). Results showed the addition of herbal extracts induced enhancement effects on BMSC-derived osteoblasts to varying extents. Ethanol extract of *Gendarussa vulgaris* induced a significant and consistent enhancement on BMSC derived osteoblast proliferation on day 7 and day 10 incubation. The enhancement percentage is 31.35% on day 7 incubation and 58.91% for day 10 incubation compared to respective negative control. Thus, we can conclude that ethanol extract of *Gendarussa vulgaris* could enhance bone-cell proliferation. Further investigation will be focused on characterization of the effects of bioactive compounds on bone cell proliferation on alkaline phosphatase and osteocalcin production.**

Keywords— **Rat BMSC, osteoblast, herbal extract, proliferation, *Gendarussa vulgaris*.**

I. Introduction

A. Background Study

Fractures caused by osteoporosis affect 50% of women and 20% of men over the age of 50 [1]. Traditional treatment of bone fracture uses autograft, allograft, vascularized fibula and iliac crest grafts [2], and other bone transport techniques but limitations exist.

A number of medicinal herbs are being investigated regarding their effectiveness in disease treatment. The survey of Barnes et al. 2002 [3] in the year 2002 on complementary and alternative medicine use among adult at United States pointed out that nineteen percent of adults in the US used natural products such as herbal medicine, functional foods, and animal-based supplements during the past 12 months prior to their survey.

Some herbs are used in bone fracture treatment as well as osteoporosis. The studies of Sun et al. 2002 and Jeong et al. 2005 [4, 5] showed that Gu-Sui-Bu has potential effects on primary and secondary bone cells culture. The herbal extract enhanced proliferation and differentiation of bone cell in the studies. On the other hand, the crude extract has been proven possessing inhibition effect on osteoclast obtained from fetal mouse long bone [6].

The two herbs and a formulation containing one of the herbs were used in this study. *Drynaria quercifolia* is one of the candidates. This plant is identified to have anti-microbial activity in the studies of Poonam et al. 2005 [7]. *D.quercifolia* was chosen in this study because it is under the same family of Gu-Sui-Bu, which has been well-established for bone healing as mentioned above. *Gendarussa vulgaris* is another locally found herb that was used in the present study. *G.vulgaris* is reported to have inhibition effect on the microbial proliferation of *Staphylococcus aureus* [8]. Neither herb has been widely studied for its potential to enhance bone-cell activity. The formulation containing *G.vulgaris* as the main constituent was provided by Mr. Lim Kok Hong a local herbalist. He claims that *G.vulgaris* (when applied to a bone fracture) results in faster healing. Further, several other local herbalists also claimed similar effects. Thus, in the present study we study these two herbal extracts and one formulation of *G.vulgaris* on the proliferation of rat BMSC derived osteoblast

Rat BMSC derived osteoblasts were used in the present study as the subject of herbal extract treatment. BMSCs are one of the favoured models used in tissue-engineering research studies, due to its plasticity and multipotent capacity. BMSC is a self-renewable source of multipotent progenitor cells with the capacity to differentiate into several distinct mesenchymal lineages, such as bone, cartilage, muscle, tendon, ligament and fat tissue [9, 10]. In bone tissue engineering, BMSC is important because it serves as osteoprogenitor cell in the skeleton system. BMSC can be induced to differentiate into osteoblast by incubating with osteogenic medium which contains dexamethasone and β-glycerolphosphate [11].

II. Methods

A. Bone marrow stromal cells isolation and cultivation

Bone marrow stromal cells (BMSCs) were isolated aseptically from tibia and femurs of male young adult Sprague Dawley rats according to the procedure described in Maniatopoulos et al. 1988 [12] with minor modifications. Following spinal dislocation and immersion in 70% alcohol for disinfection purposes, the tibia and femurs were cut from the rat. After removal of soft tissue attached to the bones, the metaphyseal ends of each excised bones were then cut off and the marrow from the midshaft was flushed. The cells were then collected in a 15 ml sterile test tube and centrifuged at 1500 rpm for 5 min. The resulting cell pellets were resuspended in 12 ml of DMEM media with 10% fetal bovine serum and plated in T-75 flasks. After 4 days incubation in a CO_2 incubator under 5% CO_2 atmosphere, at 37 °C and relative humidity of 95%, the medium was discarded and the BMSC were induced osteogenically using DMEM containing 10mM β-glycerophosphate, 50 μg mL^{-1} L-ascorbic acid and 10nM dexamethasone. The cells were subjected to an evaluation of proliferation test induced by herbal extracts after the cell reached confluency.

B. Crude extraction of the medicinal herbs

The collected herbs were dried under sunlight or in oven at 40°C. The dried herbs were weighed and ground in powder. The powder was macerated with ethanol in a ratio of 1g : 1l for 3 days. $NaSO_4$ was added to absorb water content in the mixture. The ethanol with herb powder was then filtered and evaporated with rotary evaporator at 30-35°C. The ethanolic extract was added with 200ml hexane. The mixture was left for 3 days. After that, the mixture was filtered to gain hexane extract. The residue was added with water and ethyl acetate to perform liquid-liquid extraction. Water layer was freeze-dried to obtain water extract. Meanwhile, ethyl acetate layer was evaporated to obtain ethyl acetate extract. The four types of herbal extracts were used in bone cell proliferation tests.

C. Proliferation evaluation of rat BMSC derivedosteoblasts induced by plant extracts.

Rat-BMSC-derived osteoblasts were harvested using accutase after reaching confluency. The harvested cells were then seeded in a 96well test plate at a cell density of 3000 cells/well. The test plate was incubated in a CO_2 incubator under 5% CO_2 atmosphere, at 37 °C and at a relative humidity of 95% for 24 hours. After that, the culture medium was changed to culture medium containing 100μg/ml of herbal extract. The cells cultured with culture medium alone were used as control. The cells incubated with culture medium containing 100μg/ml of herbal extract were cultured until day 3, 7 or 10. Culture medium with 100μg/ml herbal extract was changed every 2-3 days. After day 3, 7 and 10, the medium with herbal extract was discarded and replaced with fresh 100μl culture medium in each well. 10μl Cell Counting Kit-8 (Sigma) solution was added to each well. Then the test plate was incubated in incubator for 3 hours. The optical density of each well was measured at a wavelength of 460nm using a microplate reader. The OD of well incubated with herbal extract in culture medium was compared to OD of cells incubated with culture medium alone to determine the effect of herbal extract on rat BMSC derived osteoblasts.

III. Results

A. Bone marrow stromal cells isolation and cultivation.

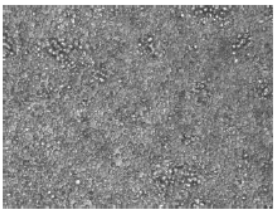


Fig. 1 Bone marrow cells plated in T-75 cm^2 immediately after harvest from rat (200x).

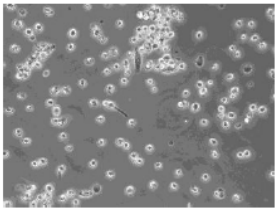


Fig. 2 Rat BMSC after 4 days

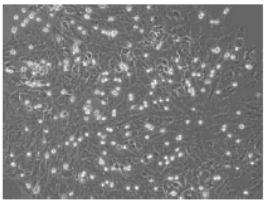


Fig. 3 Rat BMSC derived osteoblast at confluency.

The rat BMSC was cultured with primary medium (DMEM with 10% fetal bovine serum) for four days after isolation from bone. The rat BMSC was mixed with hemapoietic cells which were not attaching to surface of the bottom of the culture flask. Thus there were a lot of floating cells visible under microscope immediately after harvest from rat (Figure 1). Primary medium was discarded together with floating cells on the 4th day. Only BMSC which attached to bottom of flask remained (Figure 2). The flask

was changed to contain osteogenic medium to induce osteogenic differentiation of BMSC until the cells reached confluence (Figure 3).

B. Crude extraction of the medicinal herbs.

Four types of herbal extracts with different polarity were done for each herb in present study. Thus 12 herbal extracts were obtained from 3 herbs.

Table 1 Herbal extract and compound polarity

Herb	Extract	Compound Polarity
Drynaria quercifolia	Ethanol	70% polar + 30% non-polar
	Hexane	Non-polar
	Ethyl acetate	Polar
	Water	Semi polar
Gendarussa vulgaris	Ethanol	70% polar + 30% non-polar
	Hexane	Non-polar
	Ethyl acetate	Polar
	Water	Semi polar
Gendarussa vulgaris formulation	Ethanol	70% polar + 30% non-polar
	Hexane	Non-polar
	Ethyl acetate	Polar
	Water	Semi polar

C. Proliferation evaluation of rat BMSC derivedosteoblasts induced by plant extracts.

The proliferation of cells enhanced by herbal extracts showed increase in the optical density of test wells, as compared to control wells. The results obtained show the addition of herbal extracts induced enhancement of proliferation of rat-BMSC-derived osteoblasts to various extents. Generally, the 12 herbal extracts induced mild enhancement at day 3 of incubation. The ethanol extract of *Gendarussa vulgaris* consistently induced a significant enhancement on BMSC derived osteoblast proliferation on day 7 and day 10 incubation. The osteoblasts incubated with this herbal extract show the highest proliferation rate compared to others. The enhancement percentage is 31.35% on day 7 incubation and 58.91% for day 10 incubation compared to respective negative control.

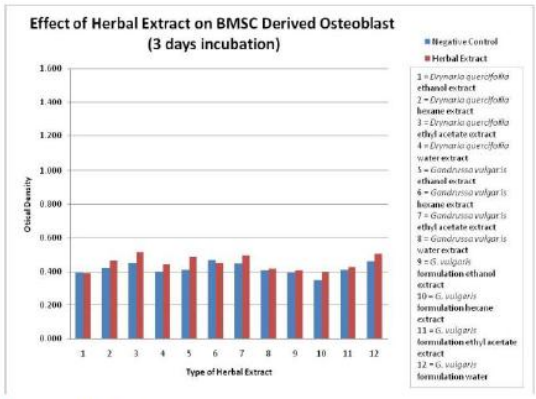


Fig. 4 Result of 3 days incubation.

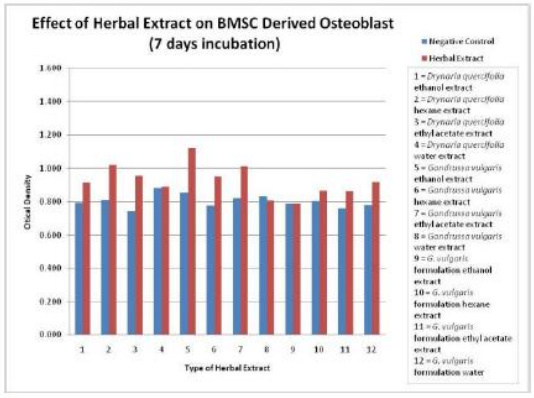


Fig. 5 Result of 7 days incubation.

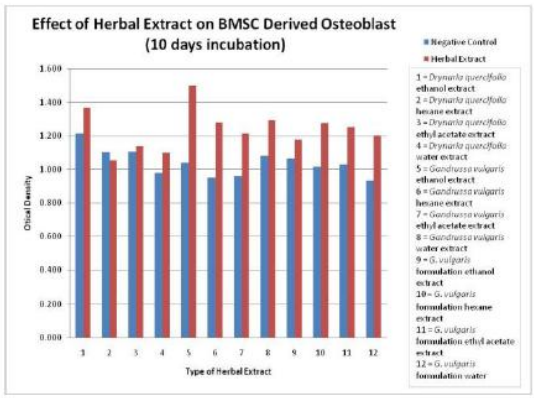


Fig. 6 Result of 10 days incubation

IV. Discussions

At present, many of the biochemical substances found in medicinal herbs have shown effectiveness in treating bone fracture. Furthermore, the side effects of natural products are considered less harmful than those of conventional medicine. The use of medicinal herbs in the treatment of bone fractures is due to enhancement of osteoblast proliferation. In the present study, enhancement of cell proliferation by ethanol extract of *Gendarussa vulgaris* is the highest among the herbal extracts, confirming the claims by the herbalist Mr Lim Kok Hong. However, others herbalist formulations of *G.vulgaris* provide less activity as compared to *G.vulgaris* alone. But formulation of combinations of other medicinal herbs for treatment of bone fracture could be due to the action of other constituents in the formulation that may enhance formation of new blood vessels and encourage bone growth factor synthesis instead of directly acting on osteoblast proliferation.

Gu-Sui-Bu (*Drynaria fortunei*) is a well-researched herb that has gained attention for its potential in bone fracture healing. It was postulated that similar compounds to those found in Gu-Sui-Bu may be found in *Drynaria quercifolia* (which was used in present study) to enhance bone cell growth.

From the results obtained, we can conclude that *G.vulgari* ethanol extract which consists of 70% polarity compounds showed an enhancement of proliferation within rat BMSC derived osteoblasts. On the other hand, the other 11 herbal extracts showed a slight enhancement. The results obtained clearly indicate the potential use of these herbs as bone fracture healing agent. However, proliferation of osteoblast is not the only parameter to measure the efficacy of a bone healing agent. Thus, further investigation on bioactive compounds on bone cell proliferation such as alkaline phosphatase and osteocalcin production need to be carried to determine the efficacy of the herbs on bone fracture healing.

References

1. Cummings S R, and Melton III L J (2002). Epidemiology and outcomes of osteoporotic fractures. Lancet. 359:1761-1767.
2. Arrington E D, Smith W J, Chambers H G, Bucknell A L, and Davino N A (1996). Complications of iliac crest bone graft harvesting. Clinical Orthopaedics. 329: 300-309.
3. Barnes Patricia M., Powell-Griner Eve, McFann Kim, and Nahin Richard L. (2002). Complementary and alternative medicine use among adults: United States. Advance Data from Vital and Health Statistics. 343:1-20.
4. Sun Jui Sheng, Lin Chun Yu, Dong Guo Chung, Sheu Shiow Yunn, Lin Feng Huei, Chen Li Ting, and Wang Yng Jiin (2002). The effect of Gu-Sui-Bu (Drynaria fortunei J. Sm) on bone cell activities. Biomaterials. 23:3377-3385.
5. Jeong Ji Cheon, Lee Jae Wook, Yoon Cheol Ho, Lee Young Choon, Chung Kang Hyun, Kim Min Gon, and Kim Cheorl Ho (2005). Stimulative effects of Drynariae Rhizoma extracts on the proliferation and differentiation of osteoblastic MC3T3-E1 Cells. Journal of Ethnopharmacology. 96:489-495.
6. Jeong Ji Cheon, Kang Sung Koo, Youn Cheol Ho, Jeong Chang Whan, Kim Hyung Min, Lee Young Choon, Chang Young Chae, and Kim Cheorl Ho (2003). Inhibition of Drynariae Rhizoma extracts on bone resorption mediated by processing of cathepsin K in cultured mouse osteoclasts. International Immunopharmacology. 3:1685-1697.
7. Poonam Shokeen, Krishna Ray, Manju Bala, and Vibha Tandon (2005). Preliminary studies on activity of *Ocimum sanctum*, *Drynaria quercifolia*, and *Annona squamosa* against *Neisseria gonorrhoeae*. Journal of the American Sexually Transmitted Disease Association. 32:106-111.
8. Paul W. Grosvenor, Agus Supriono, David O. Gray (1994) Medicinal plants from Riau Province, Sumatra, Indonesia. Part 2: antibacterial and antifungal activity. Journal of Ethnopharmacology. 45:97-111.
9. Caplan AI. (1991). Mesenchymal stem cells. Journal of Orthopaedic Research. 9:641-650.
10. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, and Marshak DR. (1999). Multilineage potential of adult human mesenchymal stem cells. Science. 284:143-147.
11. Friedenstein AJ, Piatetzky-Shapiro II, and Petrakova KV. (1966). Osteogenesis in transplants of bone marrow cells. Journal of Embryology and Experimental Morphology. 16:381-390.
12. Maniatopoulos C, Sodek J, Melcher AH. (1988). Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Research. 254:317-330.

Author: Poon Chi Tat
Institute: University of Malaya
City: Kuala Lumpur
Country: Malaysia
Email: ct_poon22@yahoo.com